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The matrix metalloproteinase matrilysin is expressed in human breast cancers as well as in normal-appearing epithelium surrounding benign and malignant breast lesions. We explored the potential role of matrilysin in mammary tumor development using a transgenic mouse approach. Multiparous female mice expressing matrilysin in the mammary glands developed hyperplastic alveolar nodules. Transgenic mice expressing both the oncogene neu and matrilysin in the mammary glands demonstrated a marked acceleration in the time of mammary tumor onset. The tumor promoting effect of matrilysin was not due to a direct effect on an erbB-related signal transduction pathway. Using a somatic cell hybridization approach, we determined that there is a dominant repressor of matrilysin expression in normal mammary epithelium that is apparently lost with malignant transformation. Although we have been unable to molecularly identify this repressor, this approach has the potential of identifying novel tumor suppressors involved in breast cancer.

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FOREWORD

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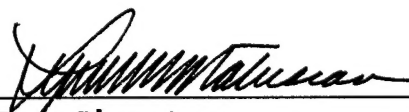
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INTRODUCTION

The matrix-degrading metalloproteinases (MMPs) have been implicated in tumor progression, invasion, and metastasis by virtue of their ability to degrade extracellular matrices. The genes for these enzymes are regulated both positively and negatively by growth factors. Our initial hypothesis was that the action of growth factors on mammary neoplasia and tumor progression is mediated, at least in part, by regulating MMP levels. The specific aims of the project were: 1) Examine the expression of matrilysin, stromelysin-1, and stromelysin-3 in transgenic mice overexpressing TGF α and/or TGF β in the mammary gland under the control of the MMTV promoter. 2) Characterize MMTV-matrilysin transgenic mice for effects of the transgene on mammary gland development and DMBA-induced tumorigenicity. 3) Examine the effects of inhibiting matrilysin levels in mammary tumorigenesis using MMTV-TIMP mice and matrilysin null mice. 4) Examine the effect of MMP alterations and TGF β on tumorigenicity in MMTV-neu transgenic mice.

The goals set forth in specific aims 2 and 4 were completed and manuscripts have been published or submitted. The manuscripts are included in the appendix and the results summarized in the "Conclusions" section. Based on the results of initial experiments, scientific rationale was presented in previous annual reports to place priority on the pursuit of the possibility that matrilysin acts to accelerate mammary tumorigenesis via a mechanism that involves the EGF/erbB pathway. These studies were completed and are included in a submitted manuscript presented in the appendix. These studies are also summarized in the "Conclusions" section.

In the last progress report, we presented studies using an in vitro culture system in which the regulation of matrilysin in breast cancer cells was examined by somatic cell fusion of early- and late-stage human breast cancer cell lines. The results of these studies indicated that a dominant-acting repressor was present in HBL100 cells. We proposed to pursue the characterization of the negative regulator of matrilysin expression since this regulator may represent a tumor suppressor that is lost in the progression of human breast cancer. We have succeeded in further characterizing a negative regulator of gene expression found in the HBL100 cells, although our attempts to clone the repressor failed. Details of these experiments are provided in the "Body" section below. A manuscript describing the characteristics of the repressor will be submitted in the near future.

PROGRESS:

Progress since the last progress report on the characterization of the negative regulator of matrilysin gene expression.

In the last annual report a brief summary was provided outlining several observations surrounding the negative regulation of matrilysin expression in somatic hybrid cells. In that report we described the construction of the somatic hybrid cells, the repression of matrilysin expression and the fact that this loss of expression was probably

not due to events involving the EGF signaling pathway. Since that report was submitted efforts have been focussed on further characterization of this hybrid cell system in an effort to understand the regulation of matrilysin and other genes in breast cancer cells.

The first set of experiments carried out were aimed at an overall characterization of the system. This involved an analysis of cellular morphology and an assessment of the ability of these hybrid cells to form tumours in nude mice. When the hybrid cells were visually compared for morphological characteristics, it was evident that the HBL 100 phenotype was a dominant trait in these cells. HBL 100 cells display a predominantly spindle or fusiform morphology as depicted in Figure 1a. MDA-MB-468 cells, on the other hand, display a predominantly epithelial or cuboidal type morphology as is clear from Figure 1b. Figure 1c shows that the hybrid cells demonstrate the HBL 100-like fusiform morphology. To confirm the dominant nature of the HBL 100 phenotype we assessed the tumorigenic properties of the HBL 100, MDA-MB-468 and hybrid cells in nude mice. Traditionally, fusion between tumorigenic and non-tumorigenic cells leads to the generation of a non-tumorigenic hybrid cell. In our hands, this general property of hybrid cells was found to be consistent. When 1×10^6 HBL 100 cells were injected subcutaneously into nude mice tumours failed to form at the site of injection. MDA-MB-468 cells, on the other hand, formed rapidly growing tumours in 5 of 5 mice when 1×10^6 cells were injected subcutaneously. When the same number of hybrid cells was injected into nude mice, tumours formed in only 3 of 5 mice, and of those tumours that grew, they demonstrated a delayed onset relative to the MDA-MB-468 cells and grew very slowly. These data are summarized in figure 2.

It was known that matrilysin expression differed between these two cell lines. Given this we sought to determine whether the expression of this gene paralleled that of the phenotypic traits discussed above. The results of a Northern blotting experiment depicted in Figure 3 reveal that the expression of matrilysin does indeed follow the dominance of the HBL 100 cells. This is the case for both constitutive (Figure 3 lanes marked control) and EGF or TA inducible expression (lanes marked EGF or TA respectively). This suggested that matrilysin expression was extinguished in the HBL 100 x MDA-MB-468 hybrid cells.

These observations, taken together, suggest that the overall phenotype of the hybrid cells (i.e., morphology and growth behaviour) parallel that of the HBL 100 cells. This is also consistent at the level of gene expression, in the case of matrilysin. Thus, in the context of a somatic cell hybrid the HBL 100 cells display dominance.

One of our long term goals was to understand the nature of the repression of matrilysin expression in the HBL 100/MDA-MB-468 hybrid cells. At first we considered known mechanisms of gene repression for our hybrid cell system. We found, however, that treatment of the cells with the potent demethylating agent 5-azacytidine or with the histone deacetylase inhibitor trichostatin A failed to reestablish matrilysin expression in the hybrid cells. Given this, we wanted to apply an expression/selection approach to clone the factor responsible for the observed repression. Our first thought was to use the matrilysin promoter driving a marker that could be selected for by virtue of its loss. This could be accomplished by having either the promoter drive expression of a fluorescent tag, such as green fluorescent protein (GFP), or a "death" gene such as thymidine kinase of herpes

simplex virus in combination with gancyclovir. In either case it was surmised that the appropriate "responder" cells could be transfected with a cDNA expression library derived from the HBL 100 cells and subsequently selected for the loss of expression of the reporter gene. Depending upon the reporter this would be accomplished through either several rounds of enrichment for cells no longer expressing GFP (with the use of a flow cytometer) or treatment of the cells with gancyclovir selecting for cells not expressing HSV-tk. Initial experiments proved this approach to be of limited sensitivity and thus, unfortunately, not applicable to our needs.

To achieve our goal we sought an alternative approach. This involved a screen for genes whose expression followed the same pattern as that of matrilysin, that is, we sought to screen for genes whose expression is specifically *repressed* as a consequence of somatic fusion. We surmised that if the hybrid cell does not represent the sum of its parts, as was suggested by our analysis of phenotype, then there ought to be genes that are expressed by either one of the parental cells but not by the hybrid generated between the two cells. With this in mind we thought that we might be able to identify a repressor by employing a surrogate gene for matrilysin. To identify such genes the following subtraction experiment was set up. mRNA derived from the hybrid cells were "subtracted" away from a pool of mRNAs derived from the two parental cell lines using the technique of Representational Difference Analysis (RDA; PCRselect, Clontech Inc.). In theory, the result should yield mRNAs expressed by either of the parental lines but not the hybrid cells, i.e., targets of repression. The technique was performed according to the manufacturer's instructions with the subtracted cDNAs being cloned into plasmid vectors and screened by northern blotting analysis. Positives (i.e., those clones that displayed a differential pattern of expression) were sequenced for identification. Of 88 clones screened, 16 represented positive "hits." Of these positives, 8 were known sequences, 4 have been identified as expressed sequence tags (ESTs) and 4 represent novel, unknown sequences. The genes identified are listed in table 1.

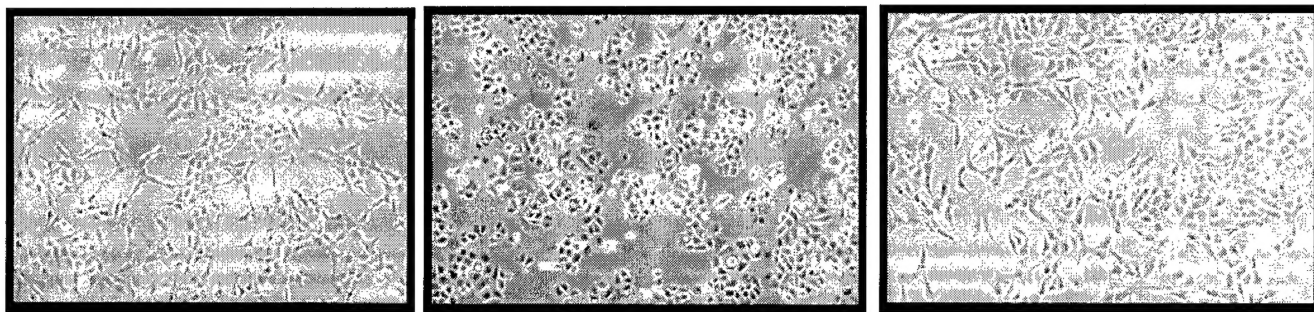
The identity of the genes discovered as differentially regulated in the hybrid cells revealed two interesting facts. The first was that all of the sequences identified as repressed in the hybrid cells were expressed by the parental MDA-MB-468 cells and not the HBL 100 cells. This is consistent with the observation that the overall phenotype of the hybrid cells was HBL 100-like and suggests that much of the dominance of the HBL 100 phenotype might come through repression of genes specifically expressed by MDA-MB-468. The second fact was that in general the *epithelial* nature of the MDA-MB-468 cells seemed to be repressed in the hybrid cells. This was characterized by the expression and subsequent hybrid mediated repression of identified genes such as Ep-CAM (GA733-2), keratin 16, keratin 19 and E-cadherin. In these hybrid cells, not only was there repression of a panel of genes, but genes associated with a "classical" epithelial phenotype. This is again consistent with our original observations with matrilysin, an MMP whose expression is generally restricted to cells of epithelial lineage.

The identification of Ep-CAM in particular as a gene repressed by somatic cell fusion allowed us now the opportunity to attempt to clone a repressor in this system by an

expression/selection approach. This is for several reasons. The first is that this gene product is abundantly expressed by the MDA-MB-468 cells and that the influence of somatic hybridization is very strong. This fact offers us a large signal-to-noise ratio when we attempt to select for its loss of expression subsequent to cDNA library transduction. The second advantage is that this is a cell surface molecule allowing us to use it as a "molecular handle." This point permits us to avoid the use of an indirect measure of gene expression again offering a highly selective and specific selection. The final reason that this molecule is beneficial is the fact that there is a number of reagents, specifically antibodies, against this molecule. Furthermore, there are antibodies conjugated to magnetic beads allowing us to use a high throughput selection approach of depletion of Ep-CAM positive cells using magnetic beads.

An attempt to clone the repressor of Ep-CAM was made by the following strategy. An HBL 100 cDNA expression library constructed in the vector pcDNA 3.1 (-) and available from Invitrogen was transfected into a population of MDA-MB-468 cells. After selection for stable transfectants (about 10,000 independent clones) these cells were then subjected to 4 rounds of depletion with Ep-CAM conjugated magnetic beads. After each round of selection, the cells remaining unbound to the beads were expanded in culture and analysed by flow cytometry. The result of this analysis is depicted in Figure 4. As evident from the figure with consecutive rounds of depletion, an Ep-CAM negative population seems to be emerging. Interestingly, this Ep-CAM negative population is also E-cadherin and matrilysin negative, consistent with our previous findings. After the fourth round of selection, clonal populations of Ep-CAM selected cells were isolated and molecularly analysed for the cDNA that they had been transfected with. Unfortunately, these cells were found to carry only empty vector, as determined by southern blot analysis (Figure 5) and PCR (data not shown), and we did not accomplish our goal of molecularly cloning the repressor. However, this attempt indicated that the depletion of Ep-CAM positive cells was a powerful strategy, one that was able to enrich for approximately one in 10,000 cells in only four repeated rounds of selection. This experiment also indicated that there might be some connection between the expression of the "epithelial" markers, Ep-CAM and E-cadherin, and the expression of matrilysin. The selected variant cells give us another tool to examine the regulation of matrilysin expression.

A final round of experiments is that of an analysis of the pattern of gene expression in heterokaryon cells. Heterokaryon cells are cells that have undergone fusion but still retain two distinct nuclei. An analysis of the patterns of gene expression in these cells will allow us to determine whether the factor(s) responsible for the observed repression are in fact genes expressed by an HBL 100 nucleus and hence, can be transferred through the cytoplasm of the heterokaryon. If this is the case, this would suggest that the repression is due to a "clonable" factor (i.e., a cDNA) and not due to alterations in chromatin structure or something that occurs in the cell as a consequence of nuclear combination. So far the experiments demonstrate that the repressor is indeed a cytoplasmic factor because fused cells with nuclei derived from both HBL 100 cells and MDA-MB-468 cells show absence of Ep-CAM staining.



A

B

C

Figure 1

Morphology of HBL 100, MDA-MB-468 and resulting fusion cells. Panel A depicts the morphology of HBL 100 cells under standard tissue culture conditions. These cells clearly show a fusiform or spindle-like appearance. MDA-MB-468 cells (panel B) show an epithelial or cuboidal appearance as is evident in their tendency to grow as clusters. The HBL 100 x MDA-MB-468 fusion cells (panel C) clearly show the dominant morphology of the HBL 100 cells.

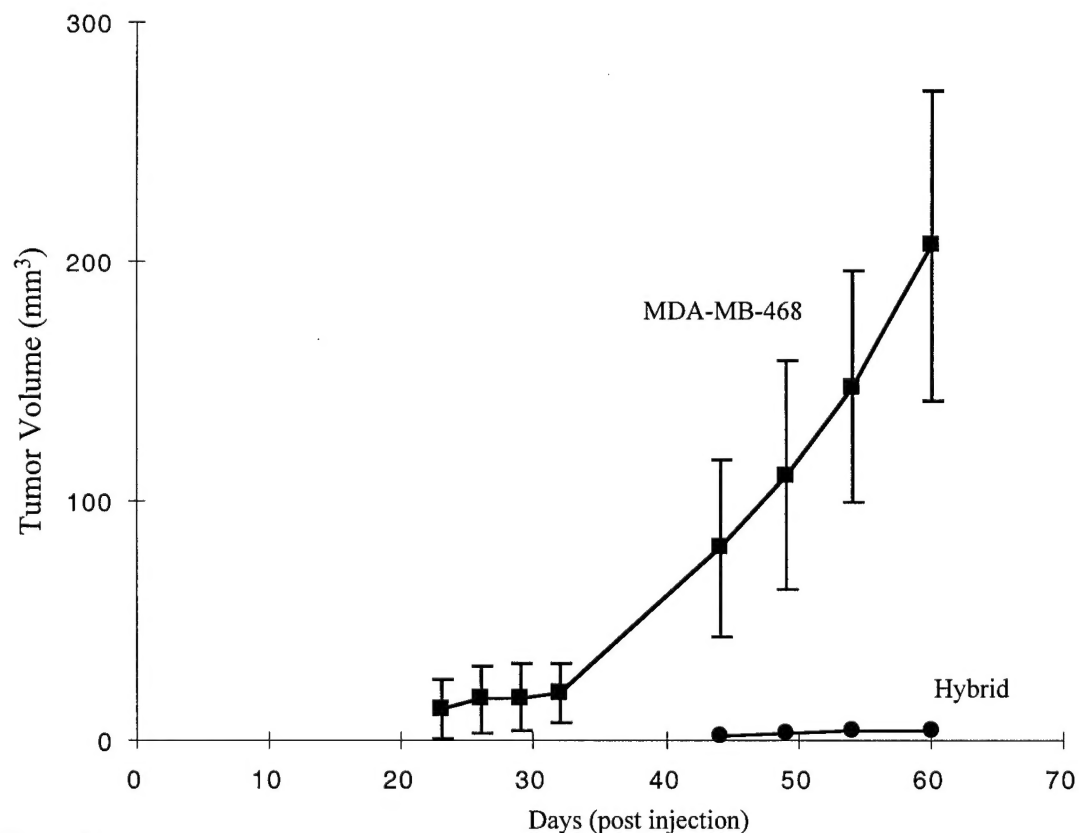


Figure 2

Tumor growth kinetics of HBL 100, MDA-MB-468 and hybrid cells in vivo. Injection of 1.0×10^6 MDA-MB-468 (■) cells resulted in onset of rapidly growing tumors at the site of injection in all (5 of 5) mice injected. Similar injection of 1.0×10^6 HBL 100 cells resulted in no observable tumor growth in all animals injected (0 of 5). Injection of 1.0×10^6 HBL 100 x MDA-MB-468 hybrid cells (●) resulted in the formation of small, slow growing tumors in only 3 or 5 animals injected.

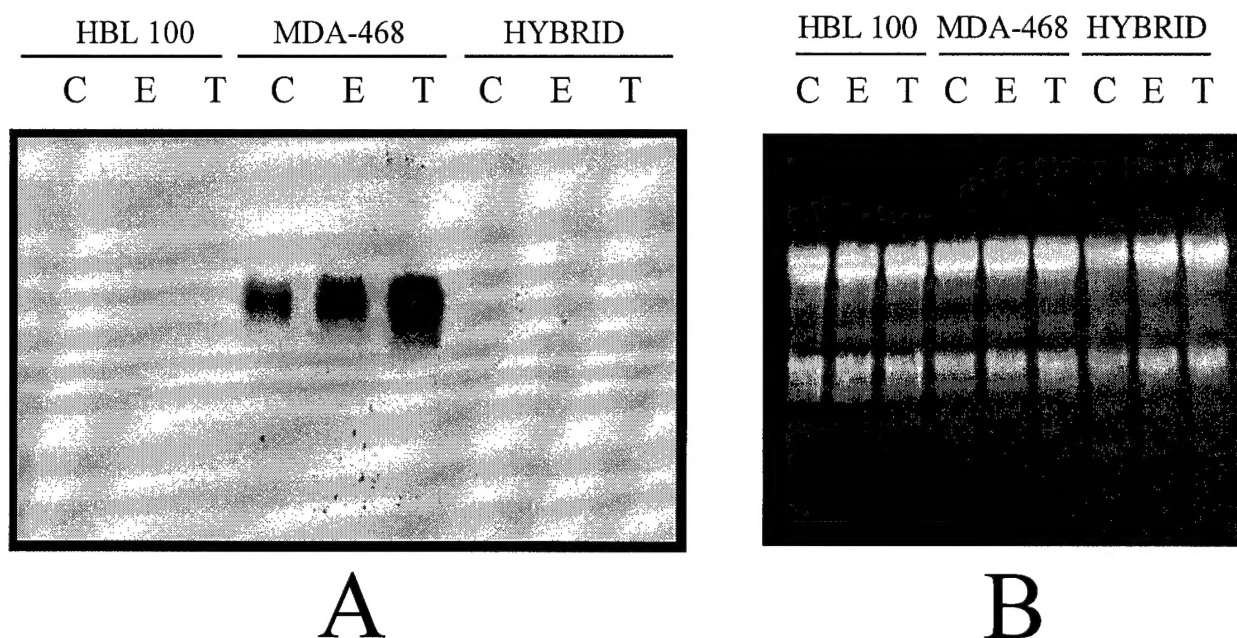


Figure 3

Matrilysin expression is repressed in HBL 100 x MDA-MB-468 hybrid cells. Matrilysin expression was analysed by Northern blotting of RNA derived from HBL 100, MDA-MB-468 and HBL 100 x MDA-MB-468 cells, either control untreated (lanes marked C), treated with 50 ng/ml of EGF (lanes marked E) or treated with 100 ng/ml of TPA (lanes marked T). As is depicted in the figure, only MDA-MB-468 cells synthesize RNA for matrilysin and this expression is inducible with either EGF or TPA. Panel B shows the ethidium bromide stained gel to indicate equal loading of the samples.

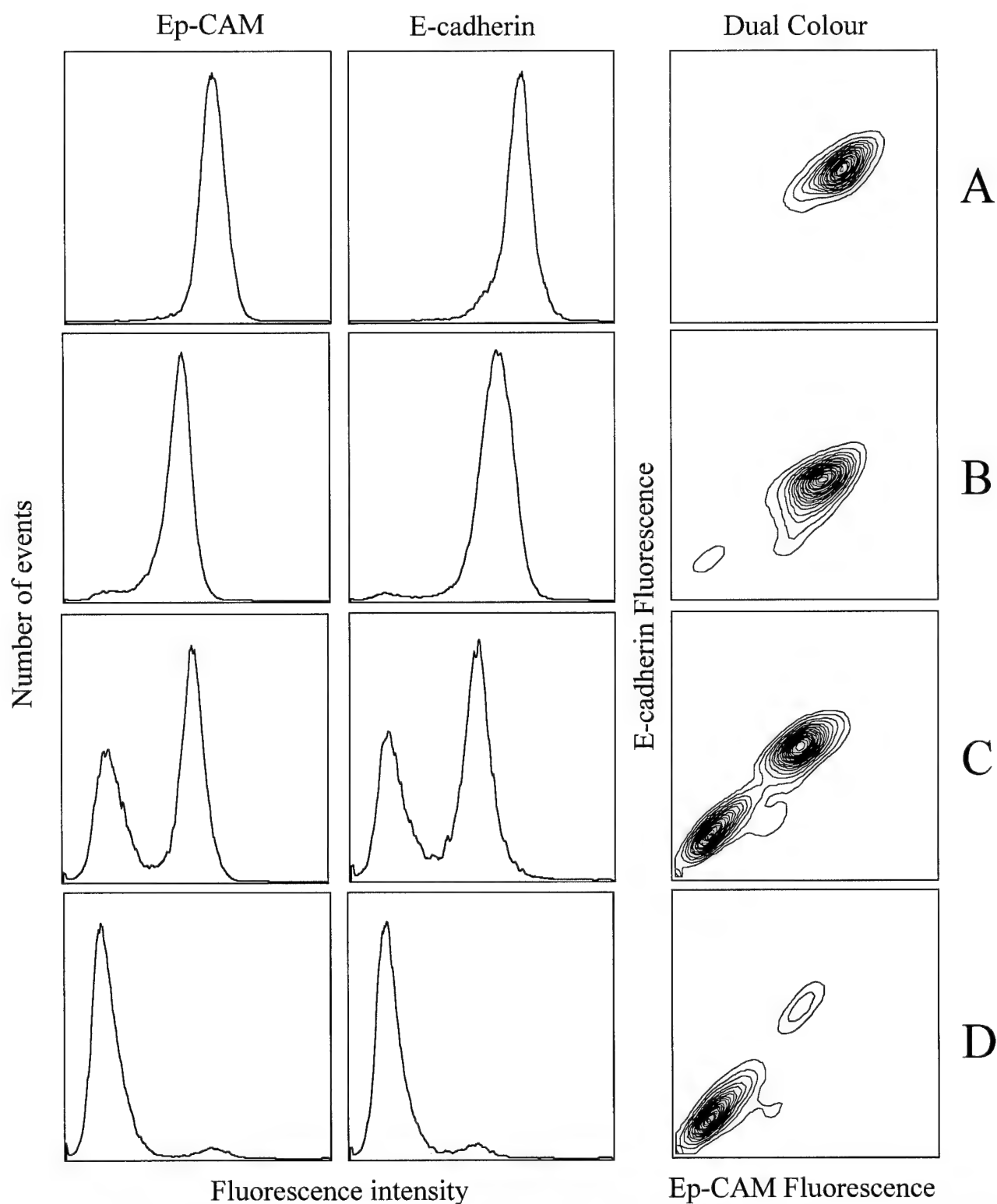


Figure 4

Flow cytometric analysis of library transfected MDA-MB-468 cells after selection for Ep-CAM negative cells using anti-Ep-CAM antibody conjugated magnetic beads. Unselected (panel A) or cells selected through 2 (panel B), 3 (panel C) or 4 (panel D) rounds of magnetic bead based depletion were analysed for Ep-CAM expression, E-cadherin expression or simultaneous expression of both antigens in a dual staining run. It is evident that after several rounds of depletion the population is being enriched for Ep-CAM negative cells. These cells also show no E-cadherin expression as is evident from the dual staining experiment.

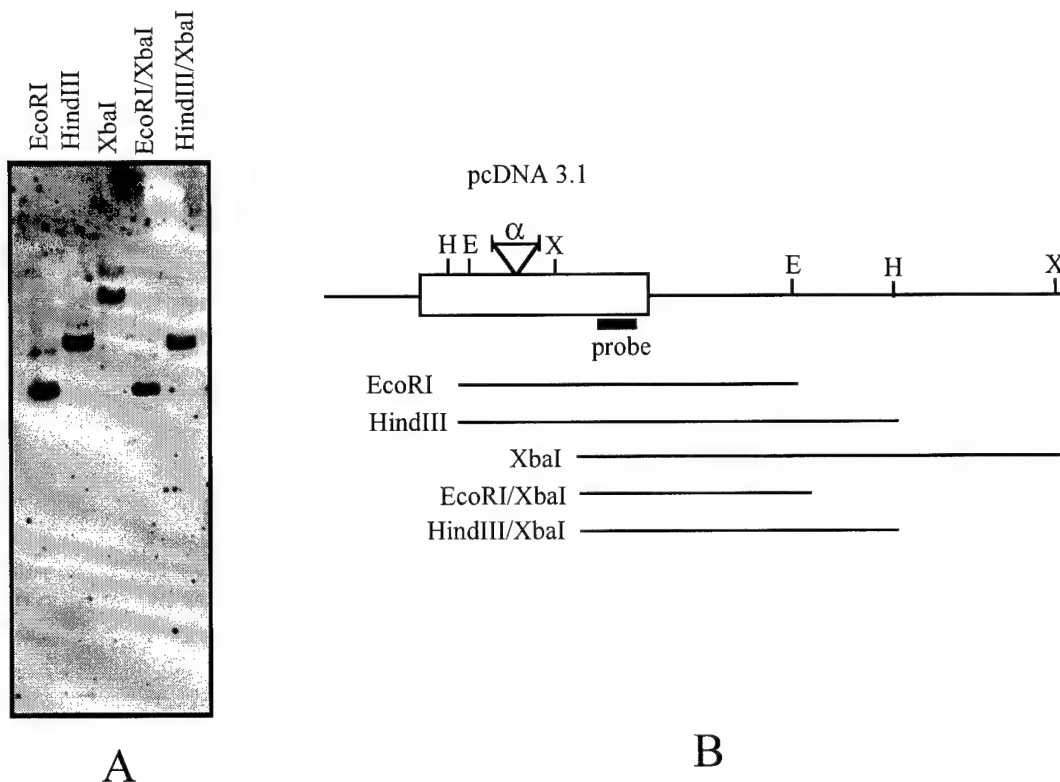


Figure 5

Southern blotting analysis indicates that the incorporated plasmid derived from the HBL 100 expression library harbours no cDNA insert. Panel A shows a Southern blot of single and double restriction endonuclease digested genomic DNA derived from clonal Ep-CAM negative cell populations. Panel B depicts a schematic representation of the locus into which pcDNA 3.1 has integrated (H=HindIII, E=EcoRI, X=XbaI). It can be deduced from the schematic that the size of the cDNA insert (α) can be given as α =E-EX or α =H-HX. The southern blot shows little if any difference between the EcoRI generated fragment and the EcoRI/XbaI generated fragment indicating the absence of a cDNA insert. Likewise, can be seen for the HindIII and HindIII/XbaI digested DNA.

CONCLUSIONS:

A. Effects of matrilysin on mammary gland development.

Transgenic mice were generated in which wildtype, constitutively active, and catalytically inactive forms of matrilysin were targeted to the mammary gland with the tissue-specific MMTV promoter. Although wildtype, active, and inactive forms of the human matrilysin protein could be produced in an in vitro culture system, mutations in the matrilysin cDNA decreased the efficiency with which the protein was produced in vivo. Therefore, animals carrying the wildtype matrilysin transgene were further examined. In contrast to previous results with MMTV-stromelysin-1 mice, mammary glands from female transgenic mice were morphologically normal throughout mammary gland development. However, both metalloproteinases resulted in mammary glands with an increased ability to produce b-casein protein in virgin animals. Matrilysin therefore induces premature mammary gland differentiation in normal mice. These results indicate that the integrity of the basement membrane can control the differentiation state of normal mammary epithelial cells, and suggest that degradation of the matrix by metalloproteinases can disrupt normal, basic cellular processes.

B. Effects of matrilysin in mammary tumor progression.

MMTV-matrilysin mice were mated with MMTV-neu transgenic mice. Bigenic MMTV-matrilysin/neu female offspring development primary mammary tumors approximately 13 weeks earlier than MMTV-neu controls. In addition, the examination of mammary glands from multiparous MMTV-matrilysin animals revealed the development of premalignant hyperplastic alveolar nodules in 50% of aged females. The presence of premalignant nodules and the accelerated development of oncogene-induced mammary tumors suggests that expression of matrilysin in the mammary epithelium contributes to early-stage tumorigenesis. Since matrilysin is found in many preneoplastic, benign, and malignant breast lesions in humans, these results indicate that matrilysin may be a target for chemopreventative or therapeutic interventions.

C. Matrilysin effects on tumor progression are not mediated through an EGF receptor pathway.

The effects of matrilysin on accelerating neu-induced mammary tumors were similar to that observed with MMTV-TGF α mice. We therefore hypothesized that the mechanism by which matrilysin might be acting could be through increasing the availability of EGF receptor ligands. However, no discernible difference in Neu receptor dimerization or activation was detected in MMTV-matrilysin/neu tumors or mammary glands compared to MMTV-neu controls. A similar percentage of MMTV-matrilysin/neu and MMTV-neu tumors acquired deletions in the cytoplasmic domain of the neu transgene, which have previously been shown to result in constitutive receptor activation. We therefore conclude that matrilysin acts as a tumor promoter in this system through a mechanism that does not involve direct activation of the erbB signal transduction pathway.

D. Identification of a dominant-acting repressor of matrilysin gene expression.

Using somatic cell hybridization we found that matrilysin expression could be the target of a dominant acting repressor. The use of various agents revealed that the repression of matrilysin was complete, in that a number of growth factors were unable to stimulate matrilysin expression. Attempts to force matrilysin expression by reversal of known repression mechanisms, through the inhibition of DNA methyltransferase or histone deacetylase, were negative and suggested a novel repressor could be identified. A cloning strategy aimed at the identification of genes whose expression was similarly repressed revealed that a number of genes, particularly "epithelial" markers were also targets of repression. Using one of these markers, Ep-CAM, an attempt to determine the factor responsible for this repression was initiated. This attempt was unsuccessful but did shed some light on the feasibility of the cloning approach and provided another system with which to study the molecular regulation of matrilysin.

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Not applicable.

APPENDICES:

5 reprints and 2 manuscripts

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Publications:

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Reviews supported in part by this grant:

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John R. MacDougall, Ph.D., postdoctoral fellow. (No salary support, but project was supported by this grant)

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Matrix Metalloproteinases in the Pathogenesis of Breast Cancer

John R. MacDougall and Lynn M. Matrisian

1. INTRODUCTION

This chapter will focus on a class of molecules, the matrix metalloproteinases (MMPs), and their role in the biological behavior of breast cancers. Following a brief overview of the characteristics of MMPs, the chapter will summarize the literature on MMPs as malignancy-associated molecules, and their role in the progression of cancers in general. The focus will then switch to the association between MMPs and breast cancer based on information regarding both spatial (i.e., *in situ* localization of MMPs in tumors) and temporal (i.e., MMP expression as a function of tumor stage and grade) patterns of MMP expression in breast cancers. The regulation of MMPs will be discussed in an attempt to understand how molecular lesions associated with the genesis and progression of breast cancer can modulate the expression and activity of MMPs. The MMPs as functional mediators of malignant behavior will next be addressed, with particular emphasis on direct lines of evidence, such as gene transfection and transgenic and knock-out approaches, to understand MMP function. This section will also draw from data analyzing the role of MMPs in the normal functioning mammary gland. Finally, the use of MMP inhibitors, both as further evidence of a functional role for MMPs in the pathogenesis of breast cancer and as potential therapeutic modalities, will be mentioned, although a more complete discussion of the application of MMP inhibitors to the clinical treatment of breast cancer is found in Chapter XX.

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by G. Sledge

2. MMPS AS MALIGNANCY-ASSOCIATED MOLECULES

2.1. The MMP Family of Molecules

The MMP family of enzymes at present is composed of at least 16 members. The members of this family of enzymes share several defining characteristics, including reliance on a metal ion (Zn^{2+}) for catalytic activity, activity at a physiological pH, and characteristic functional domains. These common domains include a propeptide, characterized by the amino acid sequence PRCGVPDV, which is responsible for latency, and the common sequence in the catalytic domain HEXGHXXGXXHS, in which the histidine residues are responsible for co-ordination of the Zn^{2+} . Other regions not common to all MMPs are the hemopexin-like and hinge region domains found in all family

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members except matrilysin, the fibronectin-like domain found in the gelatinases, and a transmembrane domain found in the membrane-type MMPs. In addition to the PRCGVPDV activation domain, it has recently been found that some MMPs have a furin cleavage site, which, in some cases, can also function as an activation signal. For a more complete review of MMP structure (see ref. 1).

NO ITALICS

As their name suggests, the MMPs degrade molecules of the basement membrane (BM) and extracellular matrix (ECM). In fact, taken as a whole, the family of MMPs is able to degrade virtually every component of the ECM. In addition, it is evident that these enzymes can act on a wide variety of substrates outside of the ECM, including growth factors and their receptors (2,3) for example. The activity of MMPs for various substrates has traditionally been the basis for the subclassification of various family members. The collagenases, composed of interstitial collagenase (MMP-1), neutrophil collagenase (MMP-8), and collagenase-3 (MMP-13) ~~are the only enzymes known to~~ degrade fibrillar type I, II, and III collagens. The gelatinases, including gelatinase A (MMP-2) and gelatinase B (MMP-9), degrade denatured collagen (i.e., gelatin), as well as type IV collagen, hence their historical name, type IV collagenases. The stromelysins, made up of stromelysin-1 (MMP-3), stromelysin-2 (MMP-10), and matrilysin (MMP-7), have the widest range of substrate specificities, including proteoglycans, laminin, fibronectin, and a variety of collagens. Other members of the MMP family for which substrate specificity has been examined include metalloelastase (MMP-12), a macrophage enzyme that degrades elastin, myelin, and other matrix proteins, and is also responsible for the generation of the angiogenesis inhibitor angiostatin (4-6); stromelysin-3 (MMP-11), which has relatively weak catalytic activity against matrix substrates (7); and the membrane-type MT1-MMP, which activates gelatinase A, as well as having activity against ECM substrates (8-10). New members of the MMP family have recently been added. MMP-18, cloned from *Xenopus*, has activity against fibrillar collagen (11); MMP-19 has been cloned from several sources, including a mammary-gland cDNA library (12,13); and enamelysin (MMP-20) is a porcine enzyme whose expression is restricted to the enamel organ (14). With considerable overlap in activity against various substrates, it has become more attractive to classify members of this family based on common protein domain structures (as discussed above, and in ref. 15). The characteristics of the MMP family members are summarized in Fig. 1., along with domain assignments.

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Fig 1

The MMP family is also associated with a family of endogenous inhibitors, the tissue inhibitors of metalloproteinases, or TIMPs. Composed of four members, TIMPs 1-4, these relatively low mol wt (approx 20-30 kDa) inhibitors are expressed by a wide variety of tissues and cell types. In general, all of the TIMPs are able to inhibit the active site of all of the MMPs, although there is evidence to suggest that there may be slight differences among various TIMP/MMP combinations (16). It has also been observed that TIMP-1 and TIMP-2 are found associated with latent gelatinase B and A, respectively. The exact function of this association remains poorly understood, although it has been speculated that, at least in the case of gelatinase A and TIMP-2, this association may facilitate molecular activation through a complex that includes MT1-MMP (17).

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Metalloproteinase	Alternative name(s)/EC designation	Domain structure
Matrilysin	MMP-7, pump-1 (EC 3.4.24.23)	PRE PRO TM CAT HEM
Interstitial Collagenase	MMP-1 (EC 3.4.24.7)	
Neutrophil Collagenase	MMP-8 (EC 3.4.24.34)	PRE PRO TM CAT HEM H HEM
Collagenase-3	MMP-13	
Stromelysin-1	MMP-3, transin (EC 3.4.24.17)	
Stromelysin-2	MMP-10, transin-2 (EC 3.4.24.22)	PRE PRO TM CAT HEM H HEM
Metalloelastase	MMP-12 (EC 3.4.24.65)	
MMP-18		PRE PRO TM CAT HEM H HEM
MMP-19		
Enamelysin	MMP-20	
Stromelysin-3	MMP-11	PRE PRO F TM CAT HEM H HEM
MT1-MMP	MMP-14	
MT2-MMP	MMP-15	PRE PRO F TM CAT HEM H HEM TM
MT3-MMP	MMP-16	
MT4-MMP	MMP-17	
Gelatinase A	MMP-2, 72kD gelatinase, IV collagenase (EC 3.4.24.24)	PRE PRO TM CAT FN HEM H HEM
Gelatinase B	MMP-9, 92kDa gelatinase, IV collagenase (EC 3.4.24.35)	PRE PRO TM CAT FN HEM CH HEM

PRE - pre-domain

PRO - pro-domain

FN - fibronectin-like domain

C - collagen-like domain

H - hinge region

F - furin cleavage site

HEM - hemopexin-like domain

TM - transmembrane domain

CAT - catalytic domain

Fig. 1. The members of the MMP family can be grouped according to domain structure. The accepted name of each MMP is given, as well as (the abbreviation used within the text). The MMP number and EC classification assigned by the enzyme commission are also given. The protein domain structure is outlined for each MMP, including the common domains, as well as domains unique to individual MMPs. MMPs -4, -5, and -6 are not shown. Initially, a collagen telopeptidase activity was assigned to MMP-4. However, this enzyme remains to be purified and unambiguously identified as a new MMP. MMP-5 and MMP-6, although initially thought novel family members at the time of discovery, were found to be MMP-2 and MMP-3, respectively.

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2.2. MMPs as Malignancy-Associated Molecules

The defining characteristics of MMPs make this family of enzymes likely candidates to be involved in tumor progression, since they are extracellular enzymes capable of matrix degradation, and are optimally active at neutral pH. It is thought that this capacity to degrade ECM allows for cellular movement and invasion in the process of metastasis (18), as well as access to growth factors liberated from degraded ECM (19).

This potential role for MMPs was first described by Liotta et al. (20), when it was discovered that highly metastatic variants of the B16 melanoma cell line displayed an enhanced ability to degrade type IV or BM collagen. Historically, this was an important observation, because, as it was thought, metastatic cancer cells must cross two or three BMs containing type IV collagen. Since then, countless studies have addressed the expression and function of virtually all of the MMP family members in a variety of tumor systems (reviewed in ref. 21).

Conclusions drawn from the literature over the past decade indicate the following general patterns for MMP involvement in tumor progression:

1. MMPs are often associated with advanced, malignant cancers, and not early, benign ones, although some interesting exceptions to this rule exist. The number of different MMPs and their relative levels also tend to increase with more aggressive stages of tumor progression. MMPs can be expressed by the malignant cells themselves, or, quite commonly, as a stromal response to the presence of the tumor.
2. MMP gene expression is controlled both positively and negatively by a number of factors also known to be involved in malignant disease progression, including growth factors, cytokines, tumor promoters and oncogenes. In addition to transcriptional regulation, both posttranscriptional and posttranslational mechanisms of MMP regulation are recognized, including the requirement for activation and subsequent susceptibility to inhibition by TIMPs.
3. Inhibition of MMP activity or expression in tumor cells leads to diminished malignancy, and MMP overexpression leads to the acquisition of a more malignant phenotype. In addition to genetic approaches to regulating MMP levels or activity, more recent studies with synthetic MMP inhibitors have not only provided proof-of-principle data for the role of MMPs in tumor aggressiveness, but also provided a vehicle for the application of this knowledge to the treatment of human disease.

Recently, understanding of the role of MMPs in neoplastic progression has taken a turn. Evidence, direct and indirect, from a variety of systems suggests that MMP expression and/or activity may also be associated with the primary growth of tumors and the competence for clonogenic growth of metastases, and not simply the requirement for an invasive phenotype to complete the metastatic journey. Evidence for this is suggested from the work of Chambers et al., who used intravital microscopy (22). In addition, experiments addressing the role of the MMP matrilysin in tumor progression have suggested that it may in fact be involved in the development of early-stage tumors (i.e., adenomas) (23), thus emphasizing the potential importance of these molecules in not only the end stage of cancer, but also the early pathogenesis of this disease. This subject has recently been reviewed by Chambers and Matrisian (24).

3. MMPS IN BREAST CANCER

3.1. Spatial and Temporal Expression of MMPs in Breast Cancer

MMP expression in breast cancer in general follows the same trends seen in other cancers. A number of MMPs have been shown to be expressed by breast cancer cells, but not expressed by their normal counterparts. These include the gelatinases A and B, membrane-type metalloproteinases, stromelysin-3, and matrilysin, and, to a lesser degree, interstitial collagenase and collagenase-3. The tissue pattern of MMP expression demonstrates an interesting, predominantly stromal pattern of expression, at least

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at the mRNA level, which has important implications for the eventual understanding of the role of MMPs in the biological behavior of breast cancer. The only MMP not showing a primarily stromal pattern of expression is matrilysin (25), which implies special consideration, and possibly unique functions, for this MMP. There are also a number of intriguing interactions between MMP subfamilies, which add to the complexity of the biology of MMPs. The MMPs of particular relevance to breast cancer are discussed individually below.

3.1.1. Stromelysin-3

The MMP stromelysin-3 was initially cloned as a gene differentially expressed in malignant, compared to fibroadenomatous, breast tissue (26). When the tissue distribution of this gene was examined, it was found to be expressed exclusively in the stroma of breast cancers, an observation which has been confirmed by several groups (27-29). This observation heralded a shift in the paradigm for MMPs in tumor progression. Until that time, MMPs had primarily been assumed to be tumor-cell associated, although some evidence for host-tumor interactions had been described (ref. 30 and references therein). A wide range of tumor cell lines expressed MMP activity, and, intuitively, if a tumor cell invaded a distant organ site, it was felt that it would have to supply its own machinery to do so. The counterintuitive spatial pattern of stromal expression has been repeated for other MMPs, in addition to stromelysin-3, and is now a common theme, although the consequences of such a pattern of expression to the progression of breast cancers remains to be clearly understood.

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Stromelysin-3 expression correlates well with the progression of breast cancers to aggressive disease. Hahnel et al. (28) found that, although only 10% of (DCIS) samples showed expression of stromelysin-3 mRNA by Northern blot analysis, approx 65% of primary and metastatic breast carcinomas showed such expression. Heppner et al. (29) noted a similar expression pattern in DCIS vs invasive cancers, and observed that, in contrast to other stromal MMPs, the pattern of hybridization for stromelysin-3 mRNA was only evident in the stromal cells directly adjacent to the tumor, rather than a diffuse staining throughout the entire tumor stroma. Wolf et al. (31) reported that *in situ* carcinomas of the comedo type, which show an increased tendency to progress toward invasiveness, stained positive for stromelysin-3 more frequently than lobular *in situ* carcinomas, which show a decreased tendency toward progression. The role of stromelysin-3 in the progression of breast cancers has been strengthened by several reports of its power as a prognostic marker in patient samples. Engel et al. (32) found, by *in situ* hybridization combined with quantitative techniques, that overexpression of stromelysin-3 was correlated with poor outcome and a shorter time to disease progression. In addition, they found that the correlation of outcome and stromelysin-3 overexpression was not correlated with other prognostic factors, including estrogen receptor status, tumor size, or microvessel counts, suggesting that stromelysin-3 expression in breast cancers could act as an independent prognostic marker.

3.1.2. Gelatinases

The gelatinase subfamily of MMPs has received considerable attention as the enzymes responsible for the type IV collagenolytic activity associated with metastatic cells. Gelatinase A levels, as measured by a variety of techniques, including gelatin

zymography, ELISA, RT-PCR, immunolocalization, and *in situ* hybridization, has been demonstrated to be elevated in breast cancer and breast cancer-derived cell lines (see ref. 33 for review).

The stage-specific expression of gelatinase A protein in breast cancer has been described by Monteagudo et al. (34), using immunolocalization techniques. Although normal tissues showed immunoreactivity restricted to myoepithelial cells of lobules and ducts, increasing progression from benign through *in situ*, invasive, and to metastatic carcinomas showed an increasing degree of tumor-cell-associated staining. Although the frequency of positive tumors did not change with increasing stage, the frequency of positive cells within a lesion did (*in situ* 10%, invasive carcinoma 80%, metastases 100%). This pattern is reminiscent of the clonal dominance model of tumor progression, in which, as a tumor cell population progresses toward metastatic competence, the tumor becomes increasingly populated by metastatically competent cells. Further studies have confirmed the tumor-cell-specific localization of gelatinase A immunostaining. For example, Hoyhtya et al. (35) found that gelatinase A staining was predominantly cytoplasmically located in tumor cells, and, in one-third of tumor samples, the localization could be found associated with the tumor cell membrane.

The mRNA *in situ* hybridization pattern of gelatinase A, in contrast to the immunolocalization pattern, has consistently been found to be associated with cells of the stroma (29,36-40). Soini et al. (38) describe moderate-to-strong hybridization seen in both fibroblasts and endothelial cells of tumors, but not the tumor cells themselves. Heppner et al. (29) report that 100% of cases examined showed stromal gelatinase A mRNA hybridization, which, in contrast to stromelysin-3, displayed a wide, diffuse pattern within the stroma of the tumor.

The immunolocalization and mRNA *in situ* analyses of gelatinase A expression suggest that, although the mRNA for gelatinase A is associated with the stromal elements within a tumor, the protein associates with the tumor cells. This generalization suggested that tumor cells might express a gelatinase receptor, in order to have localized the protein of a stromally synthesized MMP. In vitro studies confirmed this speculation, with the demonstration of a high-affinity binding site of gelatinase A on the surface of breast cancer cells (41).

Gelatinase B has also been studied in breast cancer, although not to the same extent as gelatinase A. It has been localized, like gelatinase A, to the stromal elements of the tumor, including fibroblastic cells, as well as infiltrating inflammatory cells (42), which are a common site of localization for gelatinase B. In addition, Heppner et al. (29) reported strong gelatinase B mRNA hybridization associated with the vasculature of breast cancers. In one study, however, gelatinase B mRNA could be localized to the cells of the tumor parenchyma (38). Overall expression of gelatinase B has been correlated with grade of disease; that is, aggressive disease tended to be consistently positive for gelatinase B mRNA and protein (27). The plasma levels of gelatinase B also correlated with the presence of breast cancer, compared to healthy individuals or individuals hospitalized for noncancer-related illness (43).

3.1.3. Membrane-Type Metalloproteinases

The recently described membrane-type subfamily of MMPs is characterized by their localization to the plasma membrane via a transmembrane spanning domain. The sub-

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Section 3.2.2

strate specificity of this subfamily of enzymes includes gelatins, but, most important, it seems clear that a major function for the MT-MMPs is to activate other MMPs, including gelatinase A. This interaction will be discussed further below. This has important implications for breast cancer, because, as will be discussed, one major switch in breast cancer progression is the ability of metastatic breast cancer cells to activate gelatinase A, but nonmetastatic breast cancers are unable to do so (44).

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The expression of MT-MMP mRNAs in breast cancers is also associated with stromal elements within the tumors (29,45,46). The diffuse pattern of hybridization for MT1-MMP mRNA within the tumor stroma is similar, but not identical, to the pattern observed for gelatinase A (29,45). Polette et al. (46) described the induction of MT1-MMP in fibroblasts when they were co-cultured with breast cancer cell lines. The tissue distribution of MT2- and MT3-MMPs indicates that these molecules do not play a significant role in breast cancers (47). MT4-MMP was recently cloned from a cDNA library derived from a breast carcinoma (48). This family member shows expression in a number of tumor cell lines, although the tissue distribution remains to be determined (49). It has recently been demonstrated that the immunohistological localization of MT1-MMP is associated with cells of the tumor parenchyma (47).

3.1.4. Matrilysin

Matrilysin represents an unusual member of the MMP family, because it is the only MMP that seems to be expressed by the cells of the tumor parenchyma, rather than those of the stroma (29,31). In addition, matrilysin transcripts are detected in fibroadenomas, in ductal carcinoma *in situ* specimens, and in apparently normal glandular epithelial cells adjacent to malignant lesions tissue (26,29,31). The tissue distribution and the expression of matrilysin in early lesions represents a clear divergence from the expression of the other MMPs relevant in breast cancer. These differences prompt speculation about the potential role matrilysin might have in very early stages of breast pathogenesis, as opposed to participation in late-stage tumor invasion and metastasis.

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3.1.5. Other MMPs

Heppner et al. (29) summarized the expression of nine MMPs in a small set of breast cancers, and found that, in general, most of the MMP expression represents a host response to the tumor. Interstitial collagenase and collagenase-3 were both found in a minority of the samples (4 of 13 each). However, although interstitial collagenase was found to be associated with stromal elements, the expression of collagenase-3 was found to be associated with isolated tumor cells. The expression of interstitial collagenase in a minority of cases (9 of 34) has been confirmed by Okada et al. (45). Metalloelastase (MMP-12) was also found to be expressed in a subset of cases (5 of 13), and was localized to macrophages and necrotic areas (29).

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3.2. Regulation of MMPs in Breast Cancer Cells

Under normal conditions, MMPs are a tightly regulated class of molecules that are rarely detected in adult tissues. Exceptions to this are situations in which adult tissues are undergoing dramatic morphological and functional changes, such as in the cycling and postpartum uterus, during wound healing, and postlactational mammary gland involution (50,51). In addition, since MMPs are produced as zymogens, regulation of

their activation and control of their ultimate activity by endogenous MMP inhibitors must be considered in understanding MMP regulation. The following will focus on an understanding of MMP regulation as it pertains to breast cancer.

3.2.1. Regulation of MMP Expression

The control of MMP gene expression has been studied *in vitro* in some detail. Most of the MMP gene 5' promoter sequences contain AP-1 and *ets* binding sites (reviewed in ref. 52). The presence of such promoter elements make the MMP genes responsive to oncogenic stimuli, such as *ras*, or growth factor signaling, such as EGF, via the upregulation of members of the *fos*, *jun*, and *ets* families of transcription factors. Since the presence of such oncogenic signals is a hallmark of cancer progression, it is not a surprise that MMPs can be described as malignancy-associated genes.

An example of the role of oncogenes in the regulation of MMP expression has been demonstrated by Giunciuglio et al. (53), using MCF-10A cells, a cell line derived from normal mammary epithelium, which were then infected with retroviruses harboring the *Ha-ras* oncogene and/or the *erbB-2* oncogene. In this study, the doubly transformed cells showed a higher invasive index in the Boyden chamber assay, compared to either of the single transfectants. This increase in invasive index corresponded to an increase in gelatinase A expression, as well as to a decrease in TIMP-2 expression, supporting the hypothesis that it is the balance between MMP and inhibitor expression that determines the phenotype of cancer cells. This work is reminiscent of several studies with breast cancer patients that indicate that the overexpression of gelatinase A (42,54), and, more specifically, a shift in the ratio of gelatinase A: TIMP-2 in their tumors, is associated with a poor outcome (55).

Growth factors appear to influence the expression of MMPs in breast cancer cells. Welch et al. (56) found that TGF β could stimulate the metastatic potential, and both gelatinase A and B expression and activation, in metastatic mammary carcinoma cells. Himmelstein and Muschel (57) found that co-culture of human breast cancer cells with rat embryo fibroblasts, or exposure of breast cancer cells with fibroblast-derived conditioned media, led to the induction of gelatinase B expression by the cancer cells. Korczak et al. (58) found that metastatic sublines of a murine mammary carcinoma cell line expressed approx 15-fold more stromelysin-1 and threefold less TIMP-1 than the nonmetastatic parental cells. The mechanism regulating stromelysin-1 expression in the metastatic variants was autocrine in nature, since conditioned medium from the metastatic cells, when applied to the nonmetastatic parental cells, was able to induce stromelysin-1 gene expression (59). The authors of this chapter have found a role for growth factor receptors in the regulation of MMP expression in MDA-MB-468 breast cancer cells. These cells, which overexpress the EGF-receptor (EGF-R), also constitutively express matrilysin. Sublines selected for diminished levels of EGF-R expression, through the use of antisense RNA or ligand/toxin fusion protein-mediated selection (60), show a concomitant loss of matrilysin expression, suggesting an intimate dependence of matrilysin expression on EGF-R expression and/or activity (manuscript in preparation).

Studies have also addressed the paracrine influence of breast cancer cells on the stromal expression of MMPs. Ito et al. (61) co-cultured the human breast cancer cell lines MCF-7 and BT-20 with dermal fibroblasts, and found that the former cell line

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was able to induce interstitial collagenase, gelatinase A, stromelysin-1, and TIMP-1 in the fibroblasts, and the latter cell line was able to induce gelatinase A and TIMP-1. Normal mammary epithelial cells in co-culture with fibroblasts were found to induce TIMP-1 only in the fibroblasts, suggesting that specific signals are sent from the tumor to the stromal cells, as first suggested by Biswas et al. (30 and refs. therein), and supported by *in situ* localization data (see ref. 29, for example). Further analysis in this system demonstrated that both membrane associated and soluble factors produced by the MCF-7 cells were responsible for the observed response in the fibroblasts (61). Similar results have been observed with induction of MT1-MMP in stromal fibroblasts. Polette et al. (46) found that MT1-MMP expression could be induced in fibroblasts by conditioned medium derived from MDA-231 cells, but not from MCF-7 cells. This induction was also associated with a concomitant activation of gelatinase A, as will be discussed below.

3.2.2. Regulation of MMP Activity

The MMPs are generally secreted in a zymogen form, and become activated following a conformational change that disrupts the interaction of the unpaired cysteine residue in the pro domain with the catalytic zinc (62). Activation of the cysteine switch *in vivo* is believed to occur primarily by a proteolytic cascade involving serine proteases in particular, although other mechanisms have also been suggested. MMP family members can activate other MMP family members by cleavage of the pro domain, so that a cascade of events can result in enzymatic activities capable of digestion of all components of an ECM. Stromelysin-3 is distinct from the other secreted MMPs, in that it contains a conserved furin recognition sequence, and can be secreted in an activated form following intracellular cleavage by this processing enzyme (63). Since this discovery, the MT-MMPs have also been shown to contain this domain, and MT1-MMP can be processed by furin (48,64). This observation has interesting implications for the biological consequences of the expression of these specific MMPs in tumors. For example, intracellular activation suggests the potential for a wide range of new substrates available for catalysis. Secretion of active stromelysin-3 suggests the possibility that this enzyme might be responsible for initiating a cascade of events that results in the activation of all available MMPs. This distinct activity may shed light on the observation that stromelysin-3 is an independent prognostic marker (32), and therefore presumably an important effector of the malignant phenotype of breast cancers.

Gelatinase A activation also occurs through a mechanism distinct from that of other MMPs. For gelatinase A, activation seems to require the assembly of an activation complex localized at the cell surface that is anchored by a furin-activated (i.e., intracellularly activated) MT-MMP molecule (8,65). The latent complex of gelatinase A and TIMP-2, an interaction mediated through the C-terminus of gelatinase A and the N-terminus of TIMP-2 (66,67), is thought to be localized to the cell surface by the ability of the C-terminus of TIMP-2 to bind the membrane-localized MT1-MMP (68). It is speculated that the association of these three molecules then leads to an additional MT-MMP molecule, subsequently activating the anchored gelatinase A. The assembly of this complex involving TIMP-2 may explain why this molecule, although a metalloproteinase inhibitor, has been observed to be a poor prognostic factor in breast cancers in one study (39).

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In breast cancer, the activation of gelatinase A has been a focus of much attention. This was first described by Azzam et al. (44), who reported that activation of gelatinase A, and not simply its expression, was correlated with disease aggressiveness in a large number of breast cancer cell lines, as indicated by markers such as cellular estrogen receptor negativity and vimentin positivity. Gelatinase A activation is mediated by cellular membranes (44,54,69), and the observation that the activator could be associated with the membrane fraction of cells assisted in identifying MT1-MMP as being involved in gelatinase A activation (8). The activation of gelatinase A by breast cancer cell lines could also be correlated with an epithelial-to-mesenchymal transition; i.e., the cells that showed the ability to activate gelatinase A also displayed characteristics of mesenchymal rather than epithelial cells, including the loss of cytokeratin, estrogen receptor, and E-cadherin expression, and the appearance of vimentin immunoreactivity. These markers are poor prognostic factors in breast cancers. It is intriguing that the constitutive expression of MT1-MMP was also strictly correlated with those cells that had undergone the epithelial-to-mesenchymal transition (70). These data suggest that gelatinase A activation may be a consequence of the expression of a package of genes involved in the epithelial-to-mesenchymal phenotypic transition. These genes might be co-ordinately regulated, possibly by one or only a few molecular master switches, whose oncogenic activation or inactivation might be a crucial step to the determination of malignancy in breast cancers. The regulation of MT-MMP and gelatinase A activation is far from being understood, and remains a very complex and controversial area. For example, the significance of secreted forms of MT1-MMP, the role of MT4-MMP (48), and the interaction with collagen I, which has been reported to influence the activation of gelatinase A (71), are all areas yet to be fully explored and understood.

4. MMPS AS MEDIATORS OF MALIGNANT BEHAVIOR IN BREAST CANCER

4.1. *MMPs in Cell Culture Models of Breast Cancer*

MMPs have been associated with tumor invasion and metastasis, since the observation that metastatic B16 melanoma cells demonstrate an enhanced type IV collagenase activity, compared to nonmetastatic B16 melanoma cells (20). Similar observations were made by Nakajima et al., who found that type IV collagen and lung subendothelial matrix degradation, and serum and plasma levels of gelatinase B, correlated with the metastatic potential of a series of rat mammary adenocarcinoma cells (72,73). Stromelysin-1 has also been demonstrated to influence the invasive phenotype of breast cancer cells. Cells derived from mammary tumors arising in WAP stromelysin-1 transgenic mice display an enhanced invasive ability in vitro (74). This invasive phenotype could be inhibited through the use of an MMP-specific inhibitor GM6001, but not inhibitors of other protease classes, and antisense oligodeoxynucleotides against stromelysin-1, but not collagenase-3 or stromelysin-3, were found to mimic the effect of the MMP inhibitor.

The overexpression of TIMPs in cultured cells also provides evidence for a role for MMPS in mammary tumor progression. A study by Wang et al. (75) describes transfection of the recently identified TIMP-4 into human MDA-MB-435 breast carcinoma cells. Overexpression of TIMP-4 reduced in vitro invasiveness, using the Boyden cham-

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ber assay, and in vivo metastasis to lymph nodes and lungs in nude mice. Overexpression of TIMP-4 also negatively influenced the growth of MDA-MB-435 cells, as well as angiogenesis, when assayed by histological microvessel counts.

There is growing evidence for a role for MMPs in early stages of tumor formation. The overexpression of stromelysin-3 by gene transfection in human MCF-7 breast cancer cells resulted in an enhancement in tumor growth, and, more specifically, tumor take, in immunodeficient mice (76). In addition, stromelysin-3 expression was reduced by antisense RNA technology in NIH3T3 cells that endogenously expressed stromelysin-3, reducing the tumorigenicity of these cells. In both cases, the effect was an alteration in the tumor take, and not in the growth rate of the tumor cells.

4.2. MMPs in In Vivo Models of Breast Cancer

An early clue that MMPs were a functional component of the mammary gland was the observation that type IV collagenase activity was upregulated in the involuting mammary gland, and possibly was responsible for the removal of BM in this process (77). With the mouse as the model, a great deal has been learned about the role of MMPs in mammary gland biology. In addition, advances have been made in the area of in vivo models of breast cancer, with the development of tissue-specific promoters that target the expression of oncogenes to the mammary gland.

4.2.1. MMPs in Mammary Gland Biology

The pioneering work by Bissell et al. (78) has demonstrated that the ECM contains cues important for the differentiated function of mammary epithelial cells. Given the importance of the ECM to mammary gland function, it follows that remodeling of the ECM carries with it equal importance. Talhouk et al. (79) have described the role of MMPs in the function of the normal mammary gland, and how the expression of MMPs alters the cellular interaction with the BM, which consequently has effects on cellular function. Mammary epithelial cells produce a wide range of enzymatic activities, including both gelatinolytic and caseinolytic metalloproteinases. The activity of these enzymes is lowest during lactation and highest during glandular involution when matrix is degraded and differentiated function ceases, and the gelatinolytic activity was secreted toward the basal lamina in reconstituted glands in vitro. The expression of MMPs and their inhibitors was inversely related, and tightly associated with the differentiated state of the gland, as measured by casein expression. In particular, when the level of TIMP-1 in the mammary gland was elevated through the use of slow release pellets, the high-level casein expression was prolonged, and alveolar regression was delayed, supporting the contention that MMPs and their inhibitors were responsible for controlling involution and differentiated function in the mammary gland (80).

MMPs were also considered likely effectors of the development and branching morphogenesis of the mammary gland. Witty et al. (81) found that stromelysin-1, stromelysin-3, and gelatinase A all were expressed in the developing gland, and stromelysin-1 in particular was associated with stromal fibroblasts in the elongating ducts. It is unlikely, however, that this enzyme is determining the sites of branch points as a result of its matrix-degrading activity, because stromelysin-1 expression was distributed in cleft structures, and not in end buds, and appeared to be associated with a reparative process, rather than active clearing of pathways for ductal advancement.

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These same enzymes are expressed in the mammary gland during early pregnancy, and are localized by *in situ* hybridization to the stromal components surrounding the developing alveoli of the pregnant mammary gland (81,82).

4.2.2. MMP Transgenic Mice

The generation of transgenic mice expressing MMPs, under the control of mammary-specific promoters, has been useful in the evaluation of the roles of MMPs in mammary gland function. Both the mouse mammary tumor virus long-terminal repeat (MMTV-LTR) and the whey acidic protein (WAP) promoters have been used to target expression of an activated form of stromelysin-1 to the mammary gland, and MMTV-matrilysin mice have also been generated and characterized. The differences in the regulation of these promoters, with the MMTV-promoter being regulated by hormones that appear at the time of puberty, and the WAP promoter responsive to lactational hormones, has resulted in some differences in the phenotypic presentation of these mice with respect to normal mammary gland form, function, and tumorigenesis.

4.2.2.1. MAMMARY GLAND DIFFERENTIATION AND DEVELOPMENT

The MMTV-stromelysin-1 transgenic mice generated by Witty et al. (81) expressed a phenotype referred to as "inappropriate alveolar development". At 13 wk, the glands of virgin female transgenic mice were composed of approximately twice the number of cells, compared to their nontransgenic controls, and had the morphological features of a 10-d pregnant animal. This increase in the number of cells was also associated with an increase in the frequency of cells undergoing DNA synthesis, as measured by Bromo-deoxyuridine incorporation, suggesting that expression of stromelysin-1 and the alterations in morphogenesis seen in these transgenic mice might be related to increases in cellular proliferation. Expression of κ -casein mRNA was also noted in the glands of virgin transgenic mice overexpressing stromelysin-1, indicating that expression of this gene also had an influence on cellular differentiation. Degradation of matrix components was evident by the replacement of obvious basal lamina structures with an amorphous material, as visualized by electron microscopy.

Using the WAP promoter to direct stromelysin-1 gene expression to the mammary gland, Simpson et al. (82) observed a strikingly similar phenotype to that of Witty et al. (81). Specifically, there was an enhancement of lateral branching, in addition to alveolar abnormalities. Expression of κ -casein was also observed in virgin glands of transgenic mice, emphasizing the important relationship between ECM, protease action, and cellular differentiation. During lactation, transgene expression was high, and was accompanied by alterations in BM integrity, as well as modifications in the morphology of alveoli, characterized by a reduction in size. In both pregnant and lactating glands, the expression of milk proteins was reduced, supporting the contention that cellular interaction with the ECM and BM influence the differentiated function of mammary epithelial cells. The use of the WAP-stromelysin-1 transgenic mice has further demonstrated that the reduction in gland size and cell number during lactation is through an apoptotic mechanism involving the loss of cell/ECM contact mediated through β_1 integrins (83). This was further supported through the use of TIMP-1 transgenic mice, in that the introduction of the TIMP-1 transgene into WAP-stromelysin-1 mice rescued the mammary epithelial cells from apoptosis (84). Effects of stromelysin-1 expression

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on mammary epithelial cell apoptosis were also observed in the MMTV-stromelysin-1 mice (85).

It is difficult to reconcile the observations that stromelysin-1 expression results in an increase in mammary epithelial cell proliferation, differentiation, and death, since these appear to be opposing activities. Perhaps the most palatable view of this complexity lies in the realization that the BM and ECM contains information vital to the identity and activity of the cell. Signals to grow, die, or differentiate come from the external environment, and the cell is influenced by the interface with this environment through its association with structural proteins. Thus, hormonal instructions can be perceived in a different context if normal cell-cell and cell-matrix interactions are disrupted. Further analysis of the action of MMPs in the developing mammary gland provide exceptional opportunities to understand the contributions of matrix and matrix degradation to the control of basic cellular processes such as proliferation, differentiation, and apoptosis.

4.2.2.2. TUMORIGENESIS

Using the MMTV-stromelysin-1 mice, Witty et al. (85) examined chemically induced mammary tumor formation in these animals. It was discovered that these animals showed a decreased tendency to form DMBA-induced mammary tumors. Approximately 65% of wild-type animals developed tumors, but stromelysin-1 transgenic animals developed tumors in 35% of mice. This reduction in tumor incidence was paralleled by a delay in the time to tumor onset. However, there seemed to be no significant effect of stromelysin-1 expression on tumor formation in mice that contained both the stromelysin-1 transgene and the MMTV-driven TGF- α transgene. These results suggested that the growth effect of TGF- α eliminated the inhibitory effect of stromelysin-1 on DMBA-induced mammary tumorigenesis. An evaluation of transgenic mammary glands at the time of DMBA administration revealed a 1.6-fold increase in the proliferative index of stromelysin-1-expressing mammary glands, compared to wild-type controls, and a 4-fold increase in the apoptotic index, as determined by using terminal deoxytransferase labeling techniques. These results suggested that the ectopic expression of stromelysin-1 increased the turnover rate of the mammary epithelial cells targeted by the chemical carcinogen, resulting in an elimination of a higher percentage of mutated cells through an apoptotic mechanism. The negating effect of the TGF- α transgene is apparently caused by a normalization of the ratio of proliferation:apoptosis in the bigenic mice. The results obtained by these studies are therefore likely to be influenced by the tumor induction protocol used, and appears to reflect an effect of stromelysin-1 on target cell populations, rather than an effect on cells that have already initiated tumorigenic events.

Positive effects of stromelysin-1 on mammary tumor formation have been revealed in preliminary reports that the WAP-stromelysin-1 transgenic mice develop spontaneous mammary adenocarcinomas (86-88). These mammary tumors, which have been observed in four independent lines of transgenic mice, all display reactive stromas and phenotypic abnormalities ranging from severe hyperplasia to adenocarcinomas. Tumor formation has been observed in virgin animals as young as 4 mo, suggesting that extensive hormonal fluctuations are not required for tumorigenesis.

Matrilysin has recently been targeted to the mammary gland *in vivo*, under the control of the MMTV-LTR. Although no obvious morphological consequences of matrilysin expression were observed in the development of the mammary gland, there was an enhancement of tumor formation when these animals were crossed with MMTV-*neu* animals. This enhancement displayed itself as a decreased time to tumor formation, and as a reduced frequency of disease-free animals over time, but involved no obvious differences in growth or apoptosis rates. In addition, older MMTV-matrilysin animals were observed to spontaneously develop hyperplastic alveolar nodules (HANs), but older wild-type animals did not (89). It has also been recently demonstrated that extinction of matrilysin expression, through the use of knock-out technology, leads to an approx 50% decrease in the formation of intestinal adenomas in *min* mice (23). This observation suggests that matrilysin expression is important to the formation and growth of early intestinal lesions. Experiments involving matrilysin overexpression in the mammary glands of mice leads to a similar conclusion.

5. MMPs AS THERAPEUTIC TARGETS FOR BREAST CANCER

The evidence presented so far suggests that MMPs are candidate players in the pathogenesis of breast cancer, not only at the level of invasion and metastasis, but also at earlier stages of tumor progression. This makes MMPs attractive targets for breast cancer therapies. As was discussed above, TIMPs are able to retard the invasive and growth potential of mammary tumors *in vivo* (75). Sledge et al. (90), using the synthetic MMP inhibitor BB-94, have also demonstrated that MMP inhibition can influence the growth and metastasis of breast tumors, and, in this case, the regrowth of breast tumors after surgical resection. Inhibition of mammary tumor metastasis has also been observed utilizing the same MMP inhibitor, and the effect appeared to be at the level of metastasis outgrowth (91). In an experimental model of breast cancer bone metastasis, TIMP-2 alone, or in combination with an inhibitor of bone resorption, resulted in a decrease of radiologically detectable bone lesions (92). In studies involving other tumor systems, there also seems to be a connection between MMP inhibition and tumor growth (22-24), which may be through the inhibition of tumor angiogenesis (93). If so, this would be particularly exciting in light of the results from Weidner et al. (94), in which tumor angiogenesis was strongly predictive for recurrence in women presenting with node-negative breast cancer. As clinical trials with synthetic MMP inhibitors continue, there is optimism that the low toxicities and biological effects of these compounds will prove attractive for the treatment, and perhaps even the prevention, of breast malignancies. Additional discussions of this topic can be found in Chapter XX.

6. SUMMARY AND KEY UNANSWERED QUESTIONS

This chapter has made an attempt to describe the role of MMPs as mediators of the malignant phenotype, with specific emphasis on breast cancer. Breast cancers express a variety of MMPs, and, in several cases, expression correlates with stage and grade of disease. Preclinical studies support a causal role for MMPs in several stages of tumor progression. There are, however, a number of areas in which questions remain unanswered.

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One area requiring further investigation is the tissue localization pattern of MMP expression. MMP mRNA tends to be expressed in cells within the stroma of cancers, but in only a few instances has tumor-cell-associated expression been demonstrated. It is possible that these results are influenced by limitations in the specificity and/or sensitivity of the techniques currently employed. In addition, the information ultimately required is the localization of active forms of MMP family members, a task that requires the generation of reagents not currently available. These studies, and careful genetic manipulation of stromal and tumor MMPs in animal-model systems, are required to understand the contribution of host and tumor MMPs to various stages of mammary tumor progression.

Another key area of investigation is the mechanism by which MMP expression alters the phenotype of cancer cells. It has been widely thought that MMP expression by tumor cells would enhance the invasive and metastatic capacity of cancer cells, by virtue of its ability to degrade BM and ECM components. However, recent experimental evidence suggests that MMPs may also act to modulate the growth of primary tumors and metastatic foci. The mechanism underlying this phenomenon is poorly understood, although several hypotheses are conceivable. For instance, potential substrates for MMPs could affect the activity of growth factors and cytokines, which in turn enhance tumorigenicity. These potentially novel functions for MMPs could enhance understanding of the malignant behavior of cancers, and provide new therapeutic approaches to the prevention and treatment of breast cancer.

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**The matrix metalloproteinase matrilysin influences
early-stage mammary tumorigenesis**

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ABSTRACT

Overexpression of the epithelial specific matrix metalloproteinase, matrilysin (*MAT*), has been correlated with enhanced tumorigenicity and tumor cell invasion using *in vitro* model systems. We have determined the effects of *MAT* expression on the development of mammary tumorigenesis using transgenic mice that express human *MAT* under the control of the MMTV-LTR promoter/enhancer. Examination of mammary glands from multiparous MMTV-*MAT* animals revealed the development of premalignant hyperplastic alveolar nodules in 50% of aged females. MMTV-*MAT* were mated with MMTV-*neu* transgenic mice to determine the effect of *MAT* on *neu*-induced mammary tumorigenesis. Bigenic MMTV-*MAT/neu* female offspring developed primary mammary tumors approximately 13 weeks earlier than MMTV-*neu* controls. The mechanism of enhanced *neu*-induced tumorigenesis was explored. No discernible difference in Neu receptor dimerization or activation was detected in MMTV-*MAT/neu* tumors or mammary glands compared to MMTV-*neu* controls. A similar percentage of MMTV-*MAT/neu* and MMTV-*neu* tumors acquired deletions in the cytoplasmic domain of the *neu* transgene, which have previously been shown to result in constitutive receptor activation. The presence of premalignant nodules and the accelerated development of oncogene-induced mammary tumors suggests that expression of *MAT* in the mammary epithelium contributes to early-stage mammary tumorigenesis.

INTRODUCTION

Breast cancer is the leading cause of mortality due to cancer among non-smoking women in the United States (1). Lethality is usually the result of local invasion and metastasis of neoplastic cells from the primary tumor into the underlying stroma, entry into the circulation, and growth of the cancer cells at distant sites in the body (2). Because of their ability to degrade extracellular matrix components, the matrix metalloproteinases (MMPs) have been implicated in the break-down of basement membrane and underlying stroma, thereby facilitating tumor growth, invasion, and metastasis. A causal role for MMPs in these processes has been established by studies using natural and synthetic inhibitors of MMPs (3 and references therein).

Increased expression of MMPs has been detected in various forms of mammary disease, correlating MMP expression with advancement of tumor stage (reviewed in 3). Of the MMP family members examined, matrilysin (MAT, MMP-7, pump-1, uterine metalloproteinase, EC# 3.4.24.23) is distinctive in that it is the only MMP that is expressed almost exclusively in the epithelial component of the tumor compared to the predominantly stromal expression of other MMP family members. *MAT* mRNA was detected in the neoplastic epithelial tumor cells of 70-91% of breast adenocarcinomas (5-7). The expression of *MAT* in the malignant epithelium of the colon, prostate, stomach, and lung (4) make it an ideal candidate to contribute to the invasive and metastatic phenotype of these tumors. Indeed, the overexpression of MAT in prostate tumor-derived cell lines enhances the ability of these cells to invade the diaphragm of immunodeficient mice (8). However, *MAT* mRNA has been observed in benign breast fibroadenomas (5) and in a high percentage of ductal carcinoma *in situ* specimens (7), neither of which demonstrate invasive properties. MAT mRNA and protein has also been detected in non-malignant breast epithelium (7, 9) and at low levels in the mammary glands of adult cycling female mice (10). Because *MAT* is expressed in normal, benign and malignant mammary tissues,

the presence of the MAT protein is apparently not sufficient for tumor cell invasion. In the colon, *MAT* is expressed in a high percentage of adenomas (11), and the overexpression of *MAT* in colon-derived cell lines enhances tumorigenicity, but not necessarily metastasis, following orthotopic injection into nude mice (12). In addition, we have recently demonstrated that the lack of *MAT* in a murine model of familial adenomatous polyposis reduces the incidence of benign lesions (13). Thus, *MAT* in particular may play a role in early stages of tumor progression in addition to contributing to late-stage tumor invasion and metastasis.

neu / ErbB-2 has been observed to be amplified and overexpressed in a significant number of human breast cancers (14). Neu signaling is dependent on heterodimerization with other ligand-binding ErbB receptor family members, since Neu does not directly bind ligand (reviewed in 15). Members of the epidermal growth factor (EGF) family of ligands, including EGF, transforming growth factor alpha (TGF α), heparin binding-epidermal growth factor (HB-EGF), amphiregulin, and betacellulin, can transmit this signal through their association with the EGF receptor. Similarly, binding of the heregulin family of ligands to ErbB-3 or ErbB-4 can also transmit a mitogenic signal. Several studies have shown that a high degree of *neu / ErbB-2* amplification is correlated with a poor clinical outcome (14, 16). Because of this close correlation between *neu* overexpression and mammary carcinogenesis, transgenic mice were generated that carry the native Neu protein under the control of the MMTV-LTR promoter to directly test the oncogenic potential of *neu* in mammary epithelium. Overexpression of the *neu* product in the mammary epithelium resulted in the appearance of focal mammary adenocarcinomas in approximately 70% of multiparous females by an average of 205 days that metastasized to the lungs in 72% of tumor-bearing animals (17). Although the enhancing effects of pregnancy does not accurately recapitulate the human disease, the pathological features and metastatic potential of these tumors appropriately mimic the majority of human breast cancers

(18). The development of mammary tumors in the MMTV-*neu* transgenic animals was found to be caused by various deletions within the *neu* transgene, which, although these same deletions have not yet been described in human tumors, result in the same endpoint of constitutive activation of the Neu signal transduction pathway (19). Thus, the MMTV-*neu* transgenic mice represent a reasonable model of human breast cancer based on the causative agent, the pathology, and the progression of the disease (18).

In the present studies, we attempted to recapitulate the expression of *MAT* in human breast in a mouse model system to determine the contribution of this MMP to various stages of mammary tumor progression. *MAT* was targeted to normal mammary epithelium under the control of the MMTV-LTR. The expression of *MAT* in the mammary gland had no effect on ductal branching, although production of the milk protein β -casein was observed in virgin mice (20). The MMTV-*MAT* mice show no evidence of developing palpable breast nodules in virgin or multiparous aged females. Therefore, to place *MAT* in the context of malignant breast epithelium, MMTV-*MAT* transgenic mice were mated with MMTV-*neu* animals and bigenic female offspring analyzed for mammary tumor development. Interestingly, *MAT* greatly enhanced the onset of primary mammary tumors, suggesting that *MAT* has a more extensive role in early tumor development than previously assumed. In addition, we explore the possibility that the tumor-enhancing effect of *MAT* is mediated through a Neu-related signaling pathway.

MATERIALS AND METHODS

Animal Models and Tumor Assessment. Transgenic animals expressing wildtype human *MAT* (line #3; 20) were mated to transgenic animals expressing wildtype rat *c-neu* (line #22; 17), both under the control of the MMTV promoter/enhancer. The *MAT* transgene mRNA

levels in the murine mammary gland were detectable by northern blot analysis and were therefore roughly in the same range as that observed in fibroadenomas analyzed by the same technique (6). In addition, MAT protein was detectable by immunohistochemistry in both the transgenic mice and in human breast reduction samples (9, 20), although this technique is not sufficiently quantitative to comment on the relative levels of MAT expression. Both transgenic lines were originally generated in the FVB strain of mice so that the genetic background of the parental and the double transgenic animals were identical. The resulting offspring were assayed for the inheritance of both transgenes by Southern blot analysis of genomic tail DNA using a random-primered (DNA Labeling Kit; Boehringer Mannheim, Indianapolis, IN) 1.1 kb XbaI/EcoRI fragment of human *MAT* (pG7pumpEX; 21) and a 795 bp BamHI fragment of rat *c-neu* (pMMTV-*neu*; 17).

Female transgenic animals were tested for the presence of mammary tumors by weekly palpitation beginning at approximately 18 - 20 weeks of age. Thereafter, tumors were measured with a caliper in two dimensions (length and width) on a weekly basis to monitor tumor growth rate. The formula $\text{length}/2 \times (\text{width})^2$ was used to determine the approximate volume of an elliptical tumor. The total volume of each mammary tumor was plotted against days of tumor growth and the approximate doubling time of each tumor extrapolated from the graph. Mice were sacrificed before tumor size reached 20mm in diameter. Whole mount analysis for the detection of HANs was performed on the thoracic (2nd and 3rd) and inguinal (4th) mammary glands on the left side of each animal as previously described (22).

Antibodies. Affinity purified anti-human MAT polyclonal antibody was a kind gift from Dr. William Parks, Washington University School of Medicine, St. Louis, MO (9). Rabbit polyclonal antibody to mouse casein was kindly provided by Dr. Charles Daniel, University of California at Santa Cruz, Santa Cruz, CA (23). Polyclonal antibodies against EGFR (1005), Neu

(C-18), ErbB-3 (C-17), and ErbB-4 (C-18), were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Polyclonal antibodies to phosphotyrosine were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY).

Immunohistochemistry. Paraformaldehyde-fixed, paraffin-embedded sections were analyzed as previously described (20). Sections were incubated overnight at 4°C in blocking solution with anti-human MAT primary antibody (1:1000 dilution) or control rabbit IgG (Sigma Immunochemicals, St. Louis, MO), then incubated with biotinylated anti-goat IgG (1:5000; Vector Laboratories, Burlingame, CA) for at least 1 hour at room temperature. Labeled cells were visualized using an avidin-biotin peroxidase complex (Vectastain ABC kit, Vector Laboratories) and TrueBlue peroxidase substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD). Sections were then counterstained with Contrast Red.

Immunoprecipitation and Western Blot. For analysis of total cell lysates, frozen tissue was pulverized with a mortar and pestle, homogenized in ice cold protein lysis buffer (50 mM Tris, pH 8.0; 100mM NaCl; 20 mM Hepes; 1% Triton X-100; 10mM sodium fluoride; 1 mM sodium orthovanadate; 5 µg/ml aprotinin; 5 µg/ml leupeptin, and 100 µg/ml phenylmethylsulfonyl fluoride), and incubated on ice for 20 minutes with occasional vortexing. Insoluble material was removed by centrifugation at 4°C and the supernatant collected. Protein concentrations were determined using the Bradford assay (BioRad, Hercules, CA). To precipitate specific ErbB receptors in isolation, 200 - 500 µg of cellular protein was boiled for 5 minutes in boiling buffer (0.5% SDS, 1 mM DTT, and 50 mM Tris, pH 7.4) to dissociate preformed receptor complexes and then placed on ice. Alternatively, protein lysates are directly immunoprecipitated without boiling for detection of ErbB receptor complexes. Approximately 0.5 µg of ErbB specific polyclonal antibody was then added and incubated overnight at 4°C, followed by a 1 hour incubation at 4°C with protein A-Sepharose CL-4B (Pharmacia Biotech,

Piscataway, NJ). Immune complexes were washed five times with protein lysis buffer, resuspended in 2X sample buffer (4% SDS; 200 mM DTT; 120 mM Tris, pH 6.8; 10% glycerol, and 0.02% bromphenol blue), and boiled for 10 minutes. Samples were electrophoresed through an 8.0% SDS-polyacrylamide gel and transferred to nitrocellulose membranes (MSI, Westboro, MA) for western blotting. Membranes were blocked for at least 1 hour at room temperature with TBST buffer (150mM NaCl, 10mM Tris, pH 8.0, and 0.05% Tween 20) containing 2.5% nonfat dried milk. Phosphotyrosine (1 μ g/ml) or ErbB (1:10,000 dilution) antibody was added for 2 hours at room temperature with rotation. Membranes were washed with TBST buffer and incubated with an anti-rabbit, horseradish peroxidase-conjugated secondary antibody (Promega, Madison, WI; 1:40,000 dilution) for 1 hour at room temperature with rotation. The membranes were again washed in TBST and visualized by chemiluminescence. If necessary, blots were stripped (62.5 mM Tris, pH 6.8; 2% SDS, and 100 mM β -mercaptoethanol for 30 minutes at 50°C) and reprobbed with another primary antibody.

RNAse Protection Assay. Mammary glands and mammary gland tumors were homogenized in a guanidinium/acid phenol solution, and total RNA extracted as described by Chomczynski and Sacchi (24). Antisense *neu* riboprobes and PGK-1 internal control probe was generated as previously described (19). RNAse protection assays were performed by hybridizing the above probes to 20 μ g of total RNA (25). The protected fragments were separated on a 6% denaturing gel and subjected to autoradiography. The DNA markers correspond to HaeIII-digested Φ X174 molecular weight standards (Gibco, BRL) which were end labeled with [γ -³²P]dATP.

RESULTS

Alterations in Multiparous MMTV-*MAT* Mammary Glands. Overexpression of the *MAT* transgene in MMTV-*MAT* transgenic female mice does not produce any observable morphological changes during mammary gland development (20). However, careful examination of whole mounts of representative mammary glands showed that 50% (4/8) of aged, multiparous MMTV-*MAT* wildtype transgenic females contained abnormal structures in the mammary glands (Figure 1A), while age-matched and pregnancy-matched non-transgenic mammary glands were devoid of such structures (0/8, $p=0.04$ by Fisher's Exact Test; Figure 1B). These distinctive focal areas of epithelial hyperplasia strongly resemble structures previously termed hyperplastic alveolar nodules (HANs), which are considered to be premalignant precursors that are prone to develop into mammary carcinomas (26, 27). We have not observed the appearance of palpable mammary tumors in any MMTV-*MAT* transgenic animal after 3 years of observation. The appearance of HANs in the MMTV-*MAT* mammary glands suggests that overexpression of *MAT* predisposes the mammary gland to the formation of preneoplastic lesions, but is not sufficient for the development of advanced mammary disease.

Induction of Mammary Tumors in the MMTV-*MAT* Transgenics. To investigate a potential role for *MAT* in mammary tumorigenesis, we induced mammary tumors in MMTV-*MAT* transgenic mice by mating them with MMTV-*neu* animals. The mammary glands of MMTV-*MAT/neu* and MMTV-*neu* transgenic offspring were palpated weekly to determine the onset of mammary tumors. Bigenic MMTV-*MAT/neu* animals developed mammary tumors with a morphological and histological appearance similar to those previously reported in MMTV-*neu* single transgenic animals and typical of human breast adenocarcinomas (17; Figure 2A and B). Histological examination of lung tissue from affected animals frequently revealed the presence of multiple nodular lesions lodged in pulmonary vessels (Figure 2C). These lesions

were verified as metastases originated from mammary tumors by the presence of β -casein immunoreactivity (unpublished observations).

MMTV-*MAT* female transgenic mice (line #3) express detectable human MAT protein throughout the epithelium of developing (weeks 6-14) and adult mammary glands (20). The presence of the protein produced from the *MAT* transgene in the MMTV-*MAT/neu* mammary tumors was confirmed using an anti-MAT antibody that reacts with human, but not mouse, MAT (20). The MAT protein product was detected in isolated groups of cells lying at the periphery of MMTV-*MAT/neu* mammary tumors, and not in tumors derived from MMTV-*neu* only transgenic mice (Figure 2D and unpublished data). No endogenous murine *MAT* mRNA was detected in approximately six samples of MMTV-*neu* tumors analyzed by northern blot (unpublished results).

The total number of mammary tumors per animals was similar in MMTV-*MAT/neu* compared to MMTV-*neu* animals (Table I). However, we observed a dramatic acceleration in tumor onset in MMTV-*MAT/neu* mice compared to the MMTV-*neu* control animals (Figure 3, $p < 0.00001$ by log-rank test). Fifty percent of female bigenic animals developed mammary tumors by approximately 27 weeks, while 50% of single transgenic animals developed mammary tumors by approximately 40 weeks. In addition, 100% of the MMTV-*MAT/neu* double transgenic females formed mammary tumors by 40 weeks of age, whereas 20% of the *neu* females were still tumor-free by 60 weeks of age ($p = 0.05$ by Fisher's Exact Test; Figure 3). Thus, the overexpression of *MAT* in *neu*-expressing mammary glands enhanced tumorigenesis by increasing the proportion of animals with tumors and shortening the time of tumor onset by an average of 13 weeks.

Mammary tumor growth was also monitored weekly by measuring the tumors with a caliper in two dimensions. The average doubling time of the MMTV-*neu* tumors was not significantly different than the MMTV-*MAT/neu* tumors (Table I). These data indicate that although the double transgenic mice developed mammary tumors earlier, the rate of tumor growth once established was similar between the two groups of animals.

To test the possibility that overexpression of *MAT* increases the metastatic ability of the MMTV-*neu* mammary tumor cells, we determined the percent of double transgenic and single transgenic animals with secondary lung metastases. Eighty percent of the MMTV-*neu* animals developed lung metastases, while 91% of the MMTV-*MAT/neu* double transgenic animals developed lung metastases (Table I). Thus, the overexpression of *MAT* in the MMTV-*neu* animals resulted in no statistically significant increase in the metastatic ability of the mammary tumor cells.

Function of Growth Factor Receptors in the Induction of Mammary Tumors.

The dramatic effect of *MAT* on mammary tumorigenesis raised the question of the mechanism by which MMP activity accelerates *neu*-induced mammary tumor formation. Similarly to our studies, the MMTV-*TGF α /neu* mice (28) also developed mammary tumors earlier than the MMTV-*neu* transgenics. However, unlike the MMTV-*neu* (19) mammary tumors, the MMTV-*TGF α /neu* (28) tumors do not contain deletions within the *neu* transgene, presumably due to the excess ligand available to constitutively activate the Neu signal transduction pathway. Because of the similar accelerated mammary tumor growth patterns of the MMTV-*TGF α /neu* and the MMTV-*MAT/neu* animals, we proposed that a mechanism similar to that observed in the MMTV-*TGF α /neu* animals may be operating in our system. Therefore, we hypothesized that *MAT* activity could result in an increase in the availability of soluble ErbB receptor ligands,

either through cleavage of membrane precursors or release of ligand from matrix components, thereby constitutively activating the Neu signal transduction pathway. The processing of TGF α (29) and HB-EGF (30 and references therein) to their soluble forms is mediated by MMPs, providing support for this hypothesis. In addition, several EGF receptor ligands, including HB-EGF and amphiregulin, have high affinity for proteoglycans (31, 32), and MAT is a potent proteoglycanase (33). To test this hypothesis, we first analyzed the ability of Neu to heterodimerize and activate other ErbB receptor family members in MMTV-*MAT/neu* and MMTV-*neu* mammary tumors.

Antibodies specific to the EGF receptor, ErbB-3 and ErbB-4 were used to immunoprecipitate these receptors from mammary tumor protein lysates, either as complexes or isolated molecules. The presence of Neu within pre-existing cellular complexes was then analyzed by western blotting. Immunoprecipitation with anti-EGF receptor and subsequent blotting for Neu revealed that Neu protein co-immunoprecipitated with the EGF receptor (Figure 4A for representative samples). In addition, Neu was shown to co-immunoprecipitate with ErbB-3 and ErbB-4 in the same mammary tumor protein lysates (unpublished data). However, there was no discernible difference in the association of Neu with other family member receptors in the MMTV-*MAT/neu* mammary tumors compared to the MMTV-*neu* tumors.

To determine if signaling through ErbB receptors occurred, phosphotyrosine levels (p-Tyr) of these proteins was analyzed by immunoprecipitation with anti-receptor antibody and western blotting with anti-p-Tyr (Figure 4B and C for representative samples). The EGF receptor was present and phosphorylated at moderate levels in both the MMTV-*MAT/neu* and MMTV-*neu* mammary tumor extracts (Figure 4B). High levels of Neu were also found in mammary tumors and were associated with high levels of p-Tyr in both sets of tumors (Figure

4C). ErbB-3 and ErbB-4 were also detected within the mammary tumor extracts, but were associated with very low or undetectable levels of p-Tyr (unpublished data). These data illustrate that the EGF receptor and Neu are the only ErbB receptor family members that were activated at relatively consistent levels within the mammary tumor extracts. Importantly, the level of activation of the ErbB receptors was similar between the MMTV-*MAT/neu* tumors and the MMTV-*neu* tumors, suggesting that MAT has no effect on the levels of ErbB receptor signaling in fully-developed mammary tumors.

Phosphorylation and activation of the ErbB receptors could theoretically also occur before the development of the mammary tumors. Mammary glands from MMTV-*MAT/neu* and MMTV-*neu* virgin animals at 25 - 30 weeks of age that were free of mammary tumors were processed and protein extracts examined for the presence of ErbB receptors and levels of p-Tyr. These data revealed little difference between the expression levels of the ErbB receptors, or their levels of p-Tyr between the MMTV-*MAT/neu* and the MMTV-*neu* virgin, tumor free mammary glands (unpublished data).

Constitutive activation of *neu* by small deletions in the cytoplasmic domain has been demonstrated to contribute to the development of spontaneous mammary gland tumors observed in the MMTV-*neu* animals (19). Examination of the *neu* transgene by RNase protection revealed deletions in 57% (4/7) of our MMTV-*MAT/neu* mammary tumor samples, while uninvolved mammary glands lacked any *neu* alterations (Figure 5). Similarly, MMTV-*neu* single transgenic mice develop altered *neu* message in 65% of the mammary tumors (18).

DISCUSSION

The experiments presented herein were designed to determine if *MAT* expression can contribute to mammary tumorigenesis. Human MAT protein was targeted to normal mammary epithelium, mimicking the expression of MAT in normal-appearing breast epithelium obtained from breast reductions or surrounding malignant breast lesions (7,9). Long-term exposure of transgenic mammary epithelium to *MAT* expression resulted in lesions with a strong morphological resemblance to preneoplastic HANs in aged, multiparous females, although MMTV-*MAT* tissue has not been serially transplanted to determine the potential for neoplastic conversion. Previous studies suggest that the HAN is probably derived from a single cell, but that an individual HAN population can undergo genetic changes that result in a biologically heterogeneous population of hyperplastic cells (reviewed in 27). HANs are susceptible to carcinogens, and exposure to chemical carcinogens, viruses, radiation, or exogenous hormones increases the tumor incidence of the hyperplastic cells and usually decreases the tumor latency period (27). Similarly, mating the MMTV-*MAT* animals to the MMTV-*neu* transgenics revealed a striking acceleration in the onset of mammary tumor formation by 13 weeks, representing approximately 1/3 the lifespan of these animals, accompanied by an increase in the percent of animals with tumors at a defined endpoint. Since the effects of *MAT* and HANs on tumor development is similar, it is likely that the cellular changes induced by *MAT* that result in the development of HANs are the same changes that accelerate *neu*-induced tumor formation.

The mechanism underlying the tumor-enhancing property of *MAT* expression in MMTV-*neu* mice was addressed in this study. We hypothesized that MAT activity could result in an increase in the availability of soluble ErbB receptor ligands, either through cleavage of membrane precursors or release of ligand from matrix components. We tested the role of Neu-related signal transduction in *MAT*-accelerated tumorigenesis by comparing the levels, the ability to

heterodimerize, and the activation of the ErbB receptors as determined by phosphorylation. We observed no obvious differences in these parameters in MMTV-*neu* versus MMTV-*MAT/neu* tumors or in mammary glands that were prone to develop tumors. Rather, both groups of mammary tumors contain deletions within the *neu* transgene at approximately the same frequency. Although we cannot rule out the possibility that differences in ErbB signaling are too subtle to detect with these assays or is restricted to specific states not tested in these studies, these data imply that the presence of *MAT* does not alter signaling through the ErbB family of receptors and that the effects on HANs and *neu*-induced tumor acceleration is independent of ErbB signaling.

The mechanism by which *MAT* induces HAN formation and accelerates tumor onset is not clear. The expression of *MAT* could induce hyperplastic lesions and accelerate the occurrence of *neu* deletions through an effect on cellular turn-over by altering the rate of cell proliferation and/or apoptosis. An increase in the number of epithelial cells in the population would increase the chance of a random mutation, which could generate a selective advantage for that cell and initiate the carcinogenic process. An increase in both proliferation and apoptotic indices, accompanied by morphological alterations, was observed in transgenic mice in which the MMP stromelysin-1 (STR-1) was targeted to the mammary epithelial cells (34, 35, 36). However, we observed no morphological changes in the ductal structures of MMTV-*MAT* mice, nor could we detect alterations in cellular proliferation as measured by PCNA labeling or programmed cell death as measured by the TUNEL assay (20). It is possible that the assays utilized were not sensitive enough to detect slight alterations in the proliferative or apoptotic indices of the normal or malignant mammary epithelial cells. Alternatively, the effects of *MAT* may be specific for a distinct stage of mammary physiology, perhaps explaining why premalignant HANs are observed in the glands of mice that have undergone several pregnancies. The effect has similarities to

those described for tumor promoters in that by itself, *MAT* expression induces hyperplasia but not malignant tumors and acts in conjunction with an initiating event (deletions in the *neu* transgene) to accelerate tumor development. Induction of cellular proliferation is a necessary component of tumor promotion but, not all tumor promoters show a generalized hyperplastic response (37, for example). An analysis of the cellular and molecular alterations in MMTV-*MAT* mammary epithelium and HANs may shed light on the mechanisms underlying this promotional activity.

MMTV-*neu* transgenic mice provide a reasonable model of human breast tumor progression in that they spontaneously develop adenocarcinomas with metastatic potential. The introduction of MMTV-driven *MAT* into these tumors in bigenic mice resulted in a small but not statistically significant increase in the percentage of mice with lung metastases. The lack of statistical significance may be a result of the relatively high rate of metastasis observed in the MMTV-*neu* control animals in this study. However, we also noted that the *MAT* transgene is expressed only sporadically in the periphery of advanced lesions (Figure 2D), perhaps due to the loss of differentiation characteristics in these adenocarcinomas and reduction in expression of the MMTV promoter. With these caveats, our studies provide no evidence to suggest that *MAT* plays a role in the metastatic spread of mammary adenocarcinomas.

It is not clear if the effect of *MAT* on tumorigenesis is related to a specific property of the *MAT* enzyme, or is a result of the unusual expression pattern of this MMP, i.e. its expression in the epithelial component of early lesions. Recent studies in which the stromal MMPs STR-1 and stromelysin-3 (STR-3) were expressed in normal or malignant mammary epithelial cells suggest it may be the latter. Transgenic mice expressing STR-1 under the control of the mammary epithelial cell-specific whey acidic protein (WAP) promoter spontaneously develop malignant mammary adenocarcinomas (38). The difference in the extent of the tumorigenic phenotype of

these mice compared to the MMTV-*MAT* mice (the development of malignant rather than premalignant lesions) may pertain to the differences in the promoter used, the MMP expressed, and/or the strain of the host mouse. The expression of STR-3 in human breast cancer cell lines does not increase the proliferation rate of the cells, or the metastatic capacity following injection into nude mice. However, cell lines expressing STR-3 develop tumors faster than the parental cell lines that do not express STR-3 (39). Taken together, these results suggest that MMP activity in general enhances properties of mammary epithelial cells that allow them to establish tumors.

The appearance of HANs in the MMTV-*MAT* animals and the acceleration of MMTV-*neu*-induced tumors in MMTV-*MAT/neu* transgenic mice indicates that the expression of *MAT* in the mammary epithelium contributes to early-stage mammary tumorigenesis. Although the relationship between the HANs observed in mouse models and human breast pathologies is not clear, *MAT* has been observed in some benign (5) and noninvasive (7) breast lesions, as well as in apparently normal mammary epithelium (7,9). Our results with this animal model system suggest that inhibition of MAT activity in individuals with an elevated risk for mammary carcinoma may provide a protective advantage and provide an incentive to pursue additional clinical and preclinical studies with synthetic MMP inhibitors to test the potential of this strategy in the prevention of malignant breast disease.

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FIGURE LEGENDS

Figure 1: Multiparous mammary gland phenotype in the MMTV-MAT transgenic animals. Whole mount staining of inguinal mammary glands taken from multiparous transgenic (A) and nontransgenic (B) animals. Whole mounts shown are representative of eight multiparous females animals analyzed. Note the appearance of HANs (H).

Figure 2: Histological appearance and MAT expression in MMTV-MAT/*neu* mammary tumors. A typical hematoxylin and eosin-stained section of a mammary tumor from a MMTV-MAT/*neu* animal A) 16X and B) 50X objective. C) A lung metastasis from a MMTV-MAT/*neu* animal with mammary tumors (11X objective). Several metastases were usually noted in each lung. D) Immunolocalization of human MAT in the MMTV-MAT/*neu* mammary tumors to the periphery and border of the tumor (arrows). T indicates the primary mammary tumor (50X objective).

Figure 3: Mammary tumor onset in MMTV-MAT/*neu* versus MMTV-*neu* transgenic animals. Analysis of the time to development of papable mammary tumors in double and single transgenic females. 50% of the MMTV-MAT/*neu* animals developed mammary tumors by 27 weeks, and 50% of the MMTV-*neu* animals developed mammary tumors by 40 weeks. The curves are statistically different by the log-rank test ($P < 0.00001$).

Figure 4: ErbB receptor expression in the MMTV-MAT/*neu* and MMTV-MAT mammary tumors. A) Co-immunoprecipitation of the ErbB family of receptors in MMTV-MAT/*neu* and MMTV-*neu* mammary tumor extracts. Immunoprecipitation of the EGFR, followed by western blot for the Neu protein. B - C) Immunoprecipitation and western blot analysis of ErbB receptors in mammary tumor extracts from MMTV-MAT/*neu* and MMTV-*neu* animals. Each ErbB receptor [A] EGFR and

B) Neu] was specifically immunoprecipitated from 300 μ g of mammary tumor extracts then western blotted and analyzed for the levels of phosphorylation (p-Tyr), or for the presence of the immunoprecipitated receptor to control for the loading of protein. Five to eight mammary tumor samples were assayed and four representative samples are shown for each group. N.S. = nonspecific band.

Figure 5: Altered neu transcripts are expressed in MMTV-*MAT/neu* mammary tumors. Total RNA or poly A+ selected RNA isolated from MMTV-*MAT/neu* mammary glands and mammary tumors were analyzed by RNase protection assay using an antisense rat *neu* cDNA riboprobe. The protected wildtype *neu* transcript is 60 nucleotides and protected fragments corresponding to altered transcripts (samples 9, 12, 13, and 14) are indicated with arrows. A 124 nucleotide mouse phosphoglycerate kinase (PGK-1) riboprobe was used to control for equal loading of sample RNA onto the polyacrylamide gel. Positions of DNA markers are shown on the left.

Table I: MMTV-MAT/neu vs. MMTV-neu mammary tumor characteristics

Transgenic animal	Tumor onset ^a	# tumors per animal ^b	Doubling of tumors ^b	% lung metastasis ^b
MMTV-MAT/neu	27 wks	2 ± 1 tumors	15.5 ± 6.7 days	91 (10/11)
MMTV-neu	40 wks	2 ± 1 tumors	14.0 ± 6.5 days	80 (12/15)

^a Median values representing curves that are significantly different as determined by the log-rank test; $p < 0.00001$.

^b Not statistically different as evaluated by the Student *t* test.

Figure 1

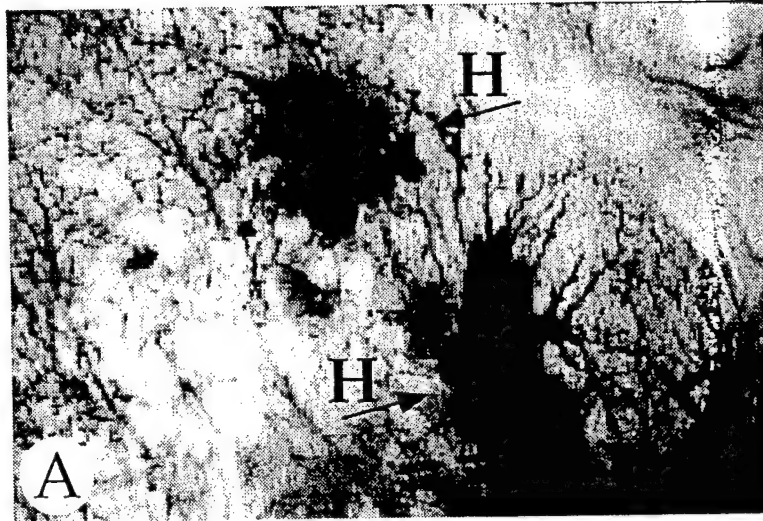


Figure 2

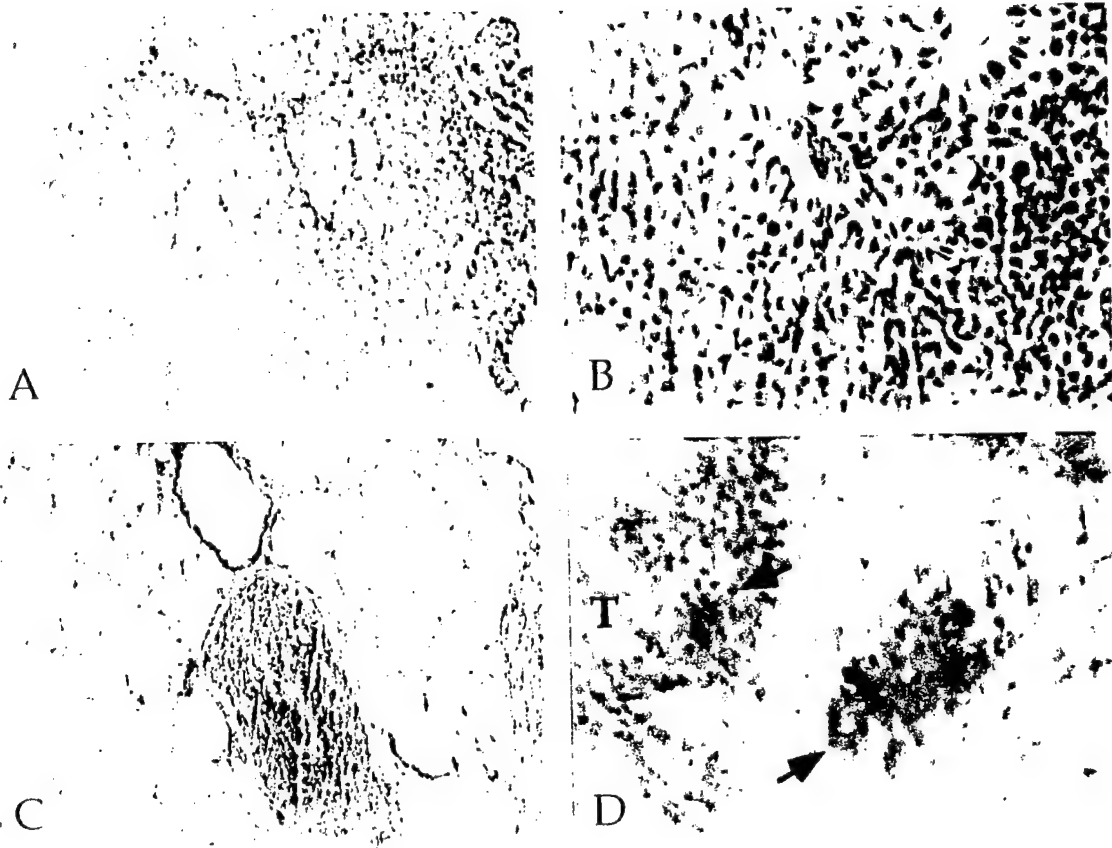


Figure 3

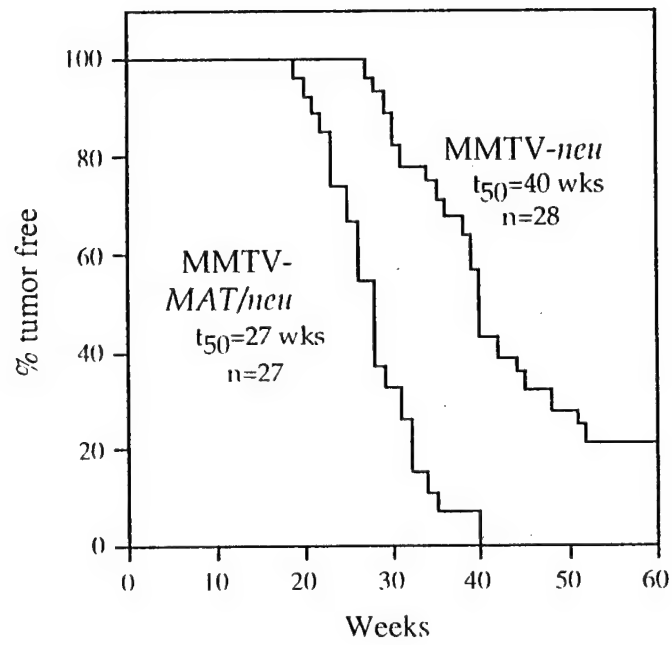


Figure 4

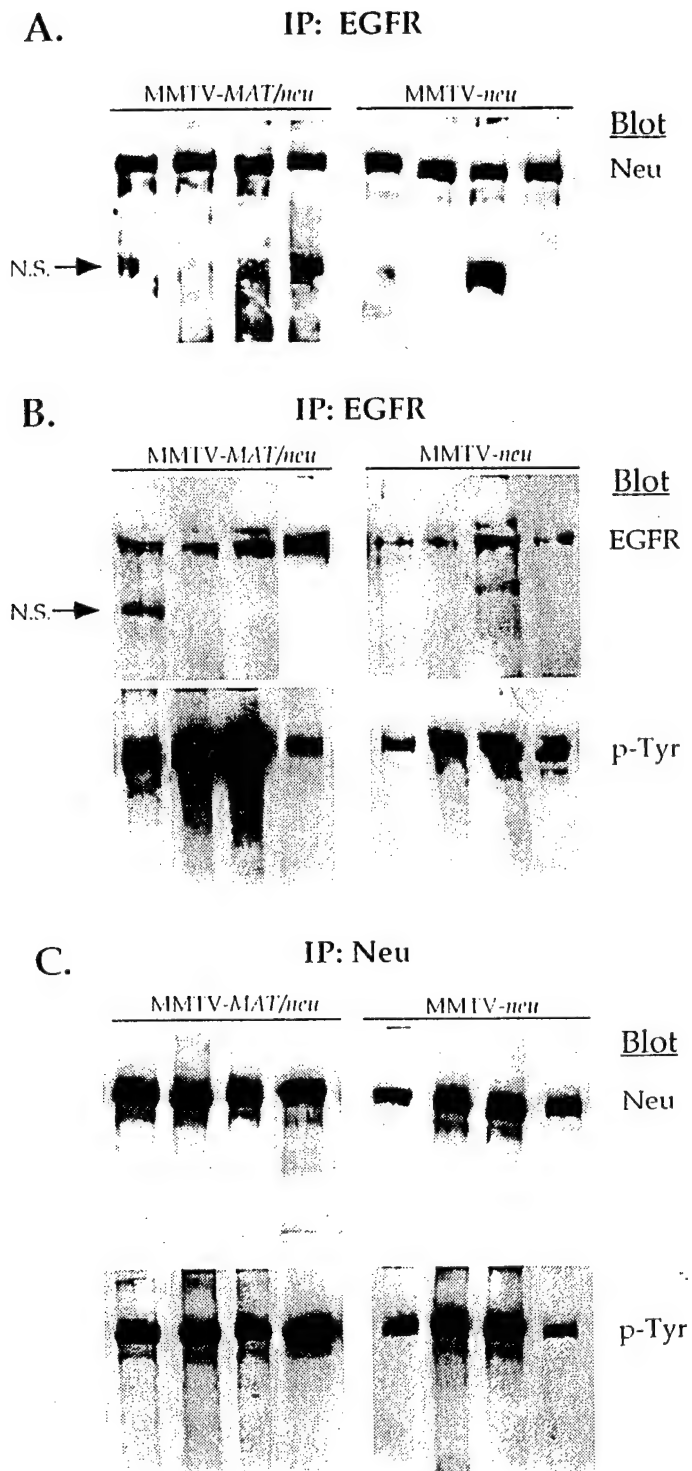
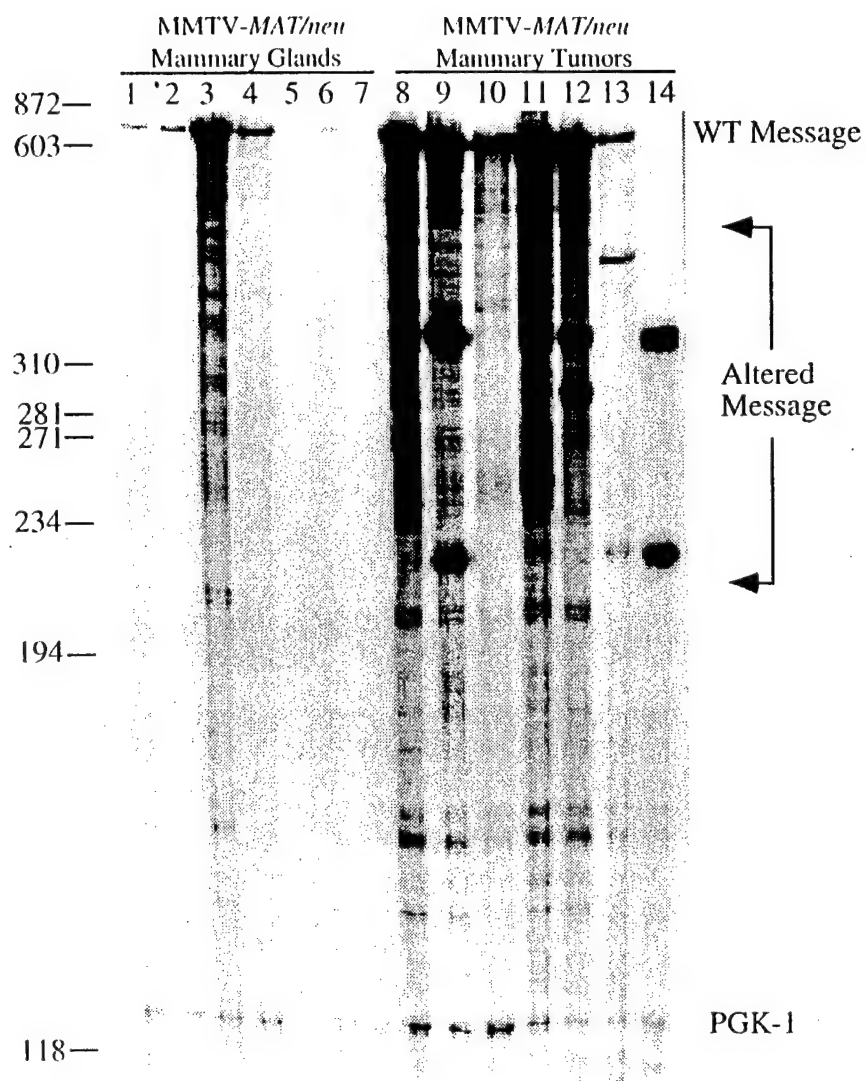


Figure 5



**The matrix metalloproteinase matrilysin influences
early-stage mammary tumorigenesis**

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Matrilysin

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I. INTRODUCTION

In the last decade, we have witnessed an explosion of knowledge about the matrix metalloproteinase (MMP) matrilysin, formerly known as pump-1 (for putative metalloproteinase-1). Although we recently provided a summary of the history and current knowledge about matrilysin (Wilson and Matrisian, 1996), many significant developments since then have led to new ideas about and reassessments of this MMP, as well as the entire family in general. Our aim in this chapter is to discuss the properties and functions of matrilysin in light of more recent experimental data and to provide our view of the picture that is emerging of matrilysin's possible roles. An in-depth description of biochemical assays for matrilysin purification and activity can be found in a review by Woessner (1995).

II. AMINO ACID SEQUENCE AND GENE ORGANIZATION

Complementary DNA encoding human matrilysin or pump-1 was first isolated by Muller *et al.* (1988) by screening a mixed tumor library in an effort to clone stromelysin-related genes. The cDNA was determined to be 49% homologous to stromelysin-1, but, at 1078 bp, it was considerably shorter than the cDNAs for stromelysin-1 and -2

and collagenase-1 (see Chapters 2 and 3). This sequence similarity, and the ability of recombinant protein to be activated with 4-aminophenylmercuric acetate (APMA) and to cleave typical metalloproteinase substrates, such as casein, fibronectin, type I, III, IV, and V gelatins, and procollagenase-1 (Quantin *et al.*, 1989), together led to the supposition that this was another, albeit unusual, member of the MMP family. Once the connection between this molecule and a rat small uterine metalloproteinase (ump) described by Woessner and co-workers was made (Sellers and Woessner, 1980; Woessner and Taplin, 1988), the stage was set for further studies to characterize this MMP. Of particular interest was the observation that the level of mRNA and enzyme activity correlated well with the time course of uterine involution and rate of proteoglycan degradation (Sellers and Woessner, 1980; Quantin *et al.*, 1989; Woessner and Taplin, 1988), suggesting that pump-1 has an active role in tissue remodeling events in the reproductive tract. This finding was exciting in that it identified another MMP family member as a potential player in complementing the degradative activity of collagenase, which is responsible for the initial cleavage of triple helical collagens. In fact, recent evidence indicates that almost all members of the MMP family are expressed during this process in rodents (Wolf *et al.*, 1996; Rudolph-Owen *et al.*, 1997a), and these enzymes appear to be important in the menstrual cycle of primates as well (Rodgers *et al.*, 1994; Brenner *et al.*, 1996; reviewed in Hulboy *et al.*, 1997).

In 1991, the Enzyme Commission named pump-1/ump matrilysin (E.C. 3.4.24.23) to describe its ability to degrade general matrix components, although other names had also been used (punctuated MMP, matrin). Matrilysin is also known as MMP-7 in the numeric designation scheme for the MMP family members. These two designations appear to be the ones used most frequently in the current literature, and "MMP-7" denotes the genetic locus for *matrilysin* in both humans (*MMP7*) and mice (*Mmp7*). Although the term matrilysin was suggested as another name for gelatinase A (Alexander and Werb, 1991), it should be noted that this name is the accepted term for MMP-7.

In addition to the cDNA clone first isolated by Muller *et al.* (1988), another clone with an additional 3' sequence was derived from a human mesangial cell cDNA library (Marti *et al.*, 1992). Both cDNA sequences aligned well with the genomic sequence (Gaire *et al.*, 1994). In addition, rat and mouse cDNA sequences have been determined (Abramson *et al.*, 1995; Wilson *et al.*, 1995). As might be expected, the mouse and rat deduced amino acid sequences are more homologous to each other (87%) than either sequence is to the human (70%). However, as

shown in Fig. 1, motifs involved in enzyme latency and activation (the PRCGV~~VDV~~ and HEXGHXXGXXH sequences) are conserved among the three species. The alignment between the rodent and human cDNA sequences does diverge at the 3' end of the coding sequence, in that there are two adjacent termination codons in the rodent cDNAs, whereas the human cDNA encodes three additional amino acids (Arg-Lys-Lys) at the corresponding position (Fig. 1). However, it was reported that recombinant human matrilysin produced in Chinese hamster ovary cells and its corresponding activated form lack these 3 carboxyl-terminal amino acids (Barnett *et al.*, 1994), which suggests that these residues do not contribute to the proteolytic activity of the enzyme.

The structures of the human and mouse genes show the same arrangement of exons (Gaire *et al.*, 1994; Wilson *et al.*, 1995). The gene is composed of six exons, the first five of which are organized in a

Mouse	(MAA) MQLTLFCFVCLLP	↓	GLALPLSQEAGDVSAHQWEQAQNYLRKFYPHDSKTK
Rat	(...) R.....RI.....C.....		E·T·L.....L.....
Human	--- MR·VL·A.....S.....P.....		GM·EL.....D·KR·LY·E··
Mouse	KVNSLVDNLKEMQKFFGLPMTGKLS	↓	PYIMEIMQKPRCGVPDVAEYSLMPNSPKW
Rat	·AT·A·K·R.....E.....RV.....		·F.....
Human	NA··EAK.....I··M·NSRVI.....		F·····
Mouse	HSRIVTYRIVSYTSDLPRIVVDQIV		KKALRMWSMQIPLNFKRVSWGTDIIIGF
Rat	··T······T····FL····R····		······
Human	T·KV······R··H·T··RL·S··N·		GKE··H·RK·V····M··
Mouse	ARRDHGDSFPFDGPGNTLGHAFA		PGPGLGGDAHFDKDEYWTDGEDAGVNFLFAA
Rat	··G····N····		······S····V··
Human	··GA····Y····A····T····		E··R····SSL·I··Y··
Mouse	THEFGHSLGLSHSSVPGTVMYPTY		QRDYSEDFSLTKDDIAGIQKLYGKRNTL---
Rat	··L····G····SS····G·H····		······K··---
Human	··L····MG··D·NA····GNGDPQN·K·		SQ··K····SNSRKK

FIG. 1. Alignment of deduced amino acid sequences of mouse, rat, and human matrilysin. The predicted amino acid sequence of mouse matrilysin is shown compared to the rat and human sequences. The symbol (·) denotes rat and human residues identical to those in the mouse sequence. The shaded areas represent residues that are identical among all three species. In the rodent sequences, initiation at the first ATG of two that are present in the 5' region of the cDNA would result in the three additional residues shown in parentheses at the amino terminus (MAA). The putative boundaries of the pre- and pro-domains are indicated by the regular and bold arrows, respectively. The conserved PRCGV~~VDV~~ and HEXGHXXGXXH motifs are underlined. The human sequence contains three additional residues at the carboxyl terminus.

manner homologous to other MMP family members for which the genomic structure is known (see Chapter 1). Although the sequence contained within the first five exons shows similarity to other MMPs, exon 6 does not have a corresponding equivalent in other MMP genes. Although exons 1 and 6 differ in size between mouse and human, the primary difference lies in the length of the introns. Southern blotting has confirmed that human matrilysin is encoded by a single gene (Gaire *et al.*, 1994), and the same is true for the mouse homologue (Wilson *et al.*, 1997). Attempts to isolate matrilysin-related genes in the mouse by low stringency Southern analysis have proven fruitless, indicating that matrilysin may exist as a unique member of the MMP family (C.L.W. and L.M.M., unpublished observations). This finding is interesting since most other MMPs, with the exception of metalloelastase, have closely related counterparts in the family. Other significant differences exist between matrilysin and the rest of the MMPs, which we will elaborate on further in this chapter.

The genes encoding human and mouse matrilysin were mapped to chromosomes 11 and 9, respectively (Knox *et al.*, 1996a; Wilson *et al.*, 1997). The q region of human Chromosome 11 contains a cluster of MMP loci, including the genes for collagenase-1, stromelysin-1, stromelysin-2 (Formstone *et al.*, 1993), and metalloelastase (Belaouaj *et al.*, 1995), in addition to matrilysin. The region of mouse chromosome 9 where the matrilysin locus (*Mmp7*) resides is syntenic with human chromosome 11q. The mouse MMP genes, like the human, may also be organized into a cluster: The matrilysin gene is closely linked to that encoding metalloelastase (Shapiro *et al.*, 1992), as well as the gene for stromelysin-1 (D.L. Hulboy and L.M.M., unpublished observations). The mouse collagenase-3 gene has been localized to chromosome 9 as well (Schorpp *et al.*, 1995), but to our knowledge has not been genetically mapped. However, the expectation is that it and at least some other MMP genes will be found at the proximal end of chromosome 9 along with matrilysin, metalloelastase, and stromelysin-1.

III. REGULATION OF EXPRESSION

A limited number of studies have been done to determine the structural elements of the matrilysin gene promoter and the effector molecules important in controlling the expression of the gene. As in most other MMP genes, the human matrilysin promoter contains a TATA box and an activator protein 1 (AP-1) site (TGAGTCA) at equivalent positions (Gaire *et al.*, 1994; reviewed in Crawford and Matrisian, 1996). In addition, there are two adjacent inverted polyomavirus enhancer A-binding protein 3 (PEA-3) elements [consensus sequence

(C/G)AGGAAG(T/C)] to which members of the *c-ets* family can bind. Although this motif occurs in several other promoters, the number, position, and orientation of the element varies considerably among MMP genes (Crawford and Matrisian, 1996; see Chapter 13). This AP-1/PEA-3 combinatorial motif appears to be the primary element in conferring responsiveness to growth factors, oncogenes, and phorbol esters (Chapter 13). The necessity of this element for expression of the matrilysin gene has been examined by functional dissection of the promoter region. Gaire *et al.* (1994) found that both the PEA-3 and AP-1 sequences are required for induction of matrilysin/CAT reporter constructs in response to the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and epidermal growth factor (EGF). Both of these molecules have been shown to up-regulate matrilysin expression in human colon adenocarcinoma cell lines (Gaire *et al.*, 1994). In addition, Sundareshan and co-workers (1997) demonstrated that TPA mediates both an increase in matrilysin expression and apoptosis in the prostate cancer cell line LNCaP; because a synthetic MMP inhibitor did not prevent TPA-induced apoptosis of these cells, the authors concluded that there is no direct relationship between matrilysin and regulated cell death under these conditions. It has also been shown that introduction of an activated *Ki-ras* oncogene into SW1417 colon carcinoma cells induces matrilysin transcription and enzymatic activity (Yamamoto *et al.*, 1995a). Using CAT assays, these workers found that an increase in AP-1 activity and high levels of AP-1 binding protein were associated with expression of the mutant *Ki-ras*. These findings led to the suggestion that induction of matrilysin expression in cells containing an activation in *ras* is mediated through an AP-1-dependent pathway. These results are also significant in light of the association of *ras* mutations with colon tumor progression; as will be discussed later, a significant majority of colorectal tumors express matrilysin.

It has been demonstrated that matrilysin is regulated by transforming growth factor β (TGF- β), potentially by elements that show similarity to the TGF- β inhibitory element (TIE) originally identified in the rat stromelysin-1 promoter (Kerr *et al.*, 1990). In general, TGF- β down-regulates expression of MMPs as it stimulates the elaboration of extracellular matrix (ECM) molecules such as fibronectin, although these effects can vary depending on factors such as cell type and state of transformation (reviewed in Massagué, 1990). Marti and co-workers (1994) demonstrated that in cultured human glomerular mesangial cells, TGF- β , suppressed both the steady-state levels of matrilysin and stromelysin mRNAs and secretion of the zymogens in a dose-dependent manner. In fact, TGF- β isoforms were identified as the principal factors

mediating the progesterone-induced inhibition of matrilysin mRNA and protein in the human endometrium (Bruner *et al.*, 1995). However, TGF- β appears to have the opposite effect on transformed cells. For example, Nakano *et al.* (1995) observed that matrilysin mRNA was up-regulated in some human glioma cell lines by this growth factor, and that, in general, TGF- β stimulated the invasive behavior of these cells in a Matrigel invasion assay. Indeed, Borchers and colleagues (1994) found that a fibroblast-derived soluble factor, possibly TGF- β , induced expression of matrilysin mRNA and protein in the human squamous cell carcinoma cell line II-4.

Other suggested candidates for the fibroblast-derived stimulatory factor found by Borchers *et al.* (1994) include PDGF and interleukins (IL) 1, 3, and 6, all of which are potentially produced by dermal fibroblasts. The promoter region of the human matrilysin gene does contain at least two sites which are homologous to the NF-IL6 consensus binding sequence (T(T/G)NNGNAA(T/G)), indicating that expression is potentially responsive to IL-1 and IL-6. In cultured human mesangial cells, matrilysin mRNA levels increased following treatment of the cells simultaneously with tumor necrosis factor α (TNF- α) and IL-1 β for 48 h (Marti *et al.*, 1992). In addition, there are other sites in the promoter region of the human matrilysin gene with similarity to consensus binding sequences for factors such as glucocorticoids, GATA-1 (required for erythroid differentiation), interferon γ (IFN- γ), and C/EBP. Indeed, Busiek *et al.* (1995) determined that matrilysin expression in cultured monocyte-derived macrophages was suppressed by glucocorticoids and IFN- γ among others. These workers also found that retinoids had an inhibitory effect, and this same effect was observed using *trans*-retinoic acid on BM314 colon cancer cells (Yamamoto *et al.*, 1995b). Unlike mesangial cells, matrilysin expression in macrophages was not influenced by IL-1, IL-6, or TNF- α . Instead, lipopolysaccharide (LPS) and opsonized zymosan, a particulate preparation of yeast cell wall material, were major stimulators of matrilysin production in these phagocytes, whereas IL-4 and IL-10 inhibited expression. Taken together, these findings emphasize that the effect of specific growth factors, cytokines, and pharmacologic agents on matrilysin expression varies depending on the cell type, its environment, and its characteristic physiologic response to inflammatory mediators.

There are several intriguing examples of matrilysin expression in isolated cells being dependent on culture conditions. As mentioned earlier, it was shown that mesangial cells synthesize a low level of matrilysin, with those levels being augmented by either TNF- α or IL-1 (Marti *et al.*, 1992). Abdel and Mason (1996) recently reported that matrilysin mRNA and protein were significantly decreased when these

cells were grown in high concentrations of glucose. Another study suggests that matrilysin expression may be influenced by cell-cell contact. The squamous carcinoma cell line II-4, which, when cocultured with fibroblasts, produces matrilysin mRNA and protein, was shown to synthesize significantly higher levels in confluent versus log phase cultures (Borchers *et al.*, 1997a). Importantly, a neutralizing antibody against E-cadherin was able to inhibit expression, as was growth in medium disruptive for E-cadherin-mediated cell-cell interactions. Although these experiments do not show a direct relationship between E-cadherin and matrilysin, they do raise the possibility of a link between these proteins which may have implications for the development and growth of neoplastic cells.

From the examples discussed, one might surmise that matrilysin expression is limited only to mesangial cells, monocyte-derived macrophages, and carcinoma cells. As will become clear in the ensuing sections of this chapter, this is not the case; however, there is a cell-type specificity in the expression of this metalloenzyme in that it is rarely produced by fibroblasts or stromal cells. To our knowledge, only two studies have shown expression of matrilysin in fibroblastic cells: Overall and Sodek (1990) reported that the lectin concanavalin A (con A) induced transcription of matrilysin mRNA in human gingival fibroblasts, which normally do not express matrilysin. This effect appeared to be mediated by changes in cell shape induced by con A. In our laboratory, we detected matrilysin mRNA in fibroblastic cells adjacent to tumor epithelium in some cases of breast carcinoma *in situ* (Heppner *et al.*, 1996). Could expression in these cells also be due to alterations in cell morphology, perhaps mediated by changes in the remodeling extracellular environment? Or could it be the result of aberrant epithelial-mesenchymal interactions in confined tumor nests? Fibroblastic expression of matrilysin was not detected in cases of invasive cancer (Heppner *et al.*, 1996) and has not been reported in other analyses of breast cancer specimens (Wolf *et al.*, 1993). Although there are no clear answers as yet, the majority of data supports the observation that matrilysin is primarily expressed by parenchymal, rather than stromal, cells.

Finally, there is one report that indicates that matrilysin expression may also be regulated at the post-transcriptional level. Wallon and co-workers (1994) showed that treatment of the human colon adenocarcinoma cell line SW1116 with an inhibitor of polyamine synthesis (difluoromethylornithine, DMFO) for several days resulted in a reduction of secreted matrilysin levels without a concomitant decrease in the steady-state level of mRNA. This reduction was thought to be due to decreased translation and/or increased degradation of the protein rather than an

alteration in the protein export process, since intracellular proenzyme did not accumulate. Overall, there is little information about the kinetics of promatrilysin synthesis and secretion. Although the zymogen lacks Asn-linked glycosylation (Crabbe *et al.*, 1992), presumably it follows an endoplasmic reticulum-mediated secretory pathway, because it contains a consensus leader sequence. However, recent evidence suggests that, at least in glandular epithelial cells and tumor cells of epithelial origin, secretion of matrilysin is directionally controlled. For example, in our laboratory, we detected immunoreactive protein on the apical side of uterine epithelial cells (Fig. 2a) and in the lumen of

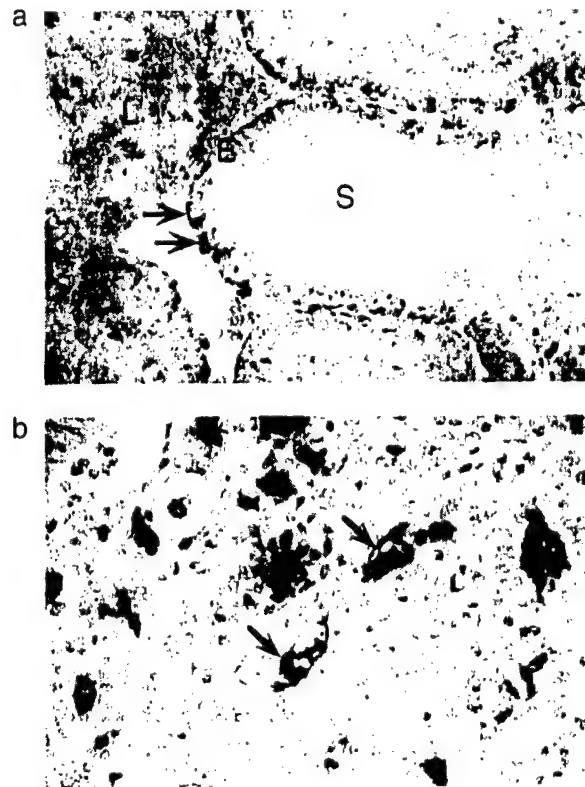


FIG. 2. Apical secretion of matrilysin protein from epithelial cells in mouse tissue. (a) A transverse section of postpartum uterus was immunostained for matrilysin using a polyclonal antibody against the carboxyl terminus. Protein can be seen on the apical side of epithelial cells (E) lining the lumen (L) of the uterus. The arrows indicate areas of intense punctate staining, which can be seen throughout the section. Luminal contents are also stained with the antibody. Protein was not detected in the adjacent stroma (S). (b) The same antibody was used to localize matrilysin in a section of a small intestinal tumor from a *Min* mouse. A significant degree of immunoreactivity is evident within the lumen (arrows) of dysplastic glands in the tumor.

dysplastic intestinal glands (Fig. 2b; Wilson *et al.*, 1997) in mice; *in situ* hybridization studies have confirmed that the mRNA is produced by the epithelial cells in these samples (Wilson *et al.*, 1995, 1997). Using a Transwell® system, Parks and co-workers (1997) showed that matrilysin is secreted apically from rat type II pneumocytes *in vitro*. However, treatment of the cells with phorbol ester resulted in nearly equivalent levels of both basal and apical secretion (Parks *et al.*, 1997). These observations indicate that the regulation of matrilysin also involves control of vectorial secretion of the proteinase and suggest that the delivery of this enzyme to different tissue compartments is associated with distinct functions.

IV. PROTEIN STRUCTURE AND PROTEOLYTIC ACTIVITIES

The ability to generate high levels of recombinant matrilysin protein has been critical for studies of the protein biochemistry and *in vitro* proteolytic activities, as well as analysis of the protein structure. The structure of the catalytic domain of matrilysin complexed with substrate-analogue inhibitors was determined (Browner *et al.*, 1995), as has been done for collagenase-1, collagenase-2, and stromelysin-1 (see Chapter 9). All show a remarkable similarity in secondary and tertiary structure. In agreement with Soler *et al.* (1994), Browner and colleagues (1995) determined that there are four metal ions bound to the matrilysin molecule, a catalytic zinc ion, a structural zinc ion, and two calcium ions. As expected, the catalytic zinc is complexed by a tetracoordinate bond among the three His residues in the HEXGHXXGXXH region. The structural zinc is ligated in a similar fashion, and binding of the calcium ions stabilizes the secondary structure. Like collagenase-1, the substrate-binding site (S_1') of matrilysin consists of a shallow hydrophobic pocket in which the amino acid residue at position 214 forms the bottom of the pocket. In contrast, stromelysin-1 and collagenase-2 are examples of MMPs containing a deep hydrophobic pocket. These differences in S_1' structure have been proposed to play a role in substrate specificity, and suggest that matrilysin and collagenase-1 may share some preferences for particular residues in the P_1' position (Welch *et al.*, 1996).

Like other MMP zymogens, promatrilysin contains an amino-terminal domain of approximately 9 kD, which is involved in maintaining its inactive state (Chapter 1). Destabilization of the complex between the cysteine in the pro-domain and the active site Zn^{2+} , primarily by stepwise cleavage of about the first 30 amino acids of the pro segment, results in a conformational change that leads to autoproteolysis and removal of the entire pro-domain. Crabbe *et al.* (1992)

determined that activation of matrilysin occurs by a similar stepwise cleavage mechanism. Like other proMMPs, the matrilysin precursor is activated by APMA, trypsin, and high temperature (53°C). The intermediates obtained appear to depend on the reagent used for activation, and include products from cleavage at Glu¹²-Leu¹³ (heat activation), Glu⁶³-Ile⁶⁴ (APMA), and Lys³³-Asn³⁴ and Arg⁶⁹-Cys⁷⁰ (trypsin). Regardless of the method used, Crabbe *et al.* (1992) found that the final autocatalytic cleavage occurs at Glu⁷⁷-Tyr⁷⁸ to yield the mature form of the protein.

Endoproteinases are believed to be the physiological activators of promatrilysin and other MMP zymogens, and plasmin is the most probable candidate, because it cleaves at sites also recognized by trypsin. Plasmin was shown to activate promatrilysin *in vitro* to about 50% of its full activity (Imai *et al.*, 1995a). In addition, there is circumstantial evidence linking serine and metal-dependent proteinases *in vivo*. For example, the promoter region of the urokinase plasminogen activator (uPA) gene contains AP-1 and PEA-3 elements, suggesting that it may be coordinately regulated with MMPs, whose genes also contain these motifs. In fact, these gene products are frequently coexpressed in remodeling and diseased tissue (e.g., see Wolf *et al.*, 1993; Airola *et al.*, 1995). A recent study by Borchers *et al.* (1997b) showed that both matrilysin and uPA were induced in the squamous cell carcinoma line II-4 when cocultured with normal melanocytes or melanoma cells. Although there is experimental evidence for processing of uPA forms by matrilysin (see later discussion), data for the reverse interaction have not been forthcoming. Therefore, other proteinases and factors yet to be identified must be involved in producing mature matrilysin. Imai *et al.* (1995a) found that stromelysin-1 and leukocyte elastase activated promatrilysin to varying degrees *in vitro*, but it is not known if these cleavage reactions occur *in vivo*.

More information is available about potential substrates for matrilysin, rather than activators of the protease. As with other MMPs, the proteolytic activities of matrilysin have been defined primarily by *in vitro* experiments using activated recombinant promatrilysin and purified substrates. Investigators have been particularly interested in determining the level of matrilysin activity against these substrates in comparison to other MMPs. Matrilysin has been found to cleave a variety of protein substrates, which can be grouped into three major categories: ECM components, proMMPs, and nonmatrix proteins. Each group is considered in more detail in the following subsections, along with some of the potential biological effects of matrilysin-mediated degradation.

A. ECM Components

Activated matrilysin has been shown to degrade the ECM proteins fibronectin (Woessner and Taplin, 1988; Quantin *et al.*, 1989), gelatins of types I, III, IV, and V (Woessner and Taplin, 1988; Quantin *et al.*, 1989), collagen type IV (Miyazaki *et al.*, 1991; Murphy *et al.*, 1991), laminin (Miyazaki *et al.*, 1991), entactin/nidogen (Mayer *et al.*, 1993; Sires *et al.*, 1993), fibulin-1 and -2 (Sasaki *et al.*, 1996), elastin (human matrilysin only; Murphy *et al.*, 1991), vitronectin (Imai *et al.*, 1995b), the cartilage proteoglycan aggrecan (Sellers and Woessner, 1980; Fosang *et al.*, 1992), cartilage link protein (Nguyen *et al.*, 1993), the chondroitin sulfate proteoglycan versican (Halpert *et al.*, 1996), and tenascin-C isoforms (Siri *et al.*, 1995). Matrilysin digests fibronectin more efficiently than stromelysin-1 (Woessner and Taplin, 1988; Quantin *et al.*, 1989), but does not hydrolyze collagen IV at the same rate (Murphy *et al.*, 1991). Furthermore, it was determined that matrilysin has little, if any, activity against native fibrillar collagens, whereas stromelysin-1 is at least able to degrade collagen III to a limited extent (Quantin *et al.*, 1989). Sires *et al.* (1993) showed that matrilysin cleaves entactin/nidogen, a glycoprotein that links laminin and collagen type IV, from 100- to 600-fold more rapidly than either collagenase-1 or gelatinase B. Matrilysin attacks the link region between two of the major globular domains, as well as several sites within these domains (Mayer *et al.*, 1993; Sires *et al.*, 1993). Although stromelysin-1 also cleaves entactin/nidogen, the majority of the sites do not appear to overlap with those found for matrilysin (Mayer *et al.*, 1993). Fibulin-1 and -2, which are found in basement membranes and in fibronectin-rich fibrils, are also sensitive to matrilysin, according to a recent report by Sasaki *et al.* (1996). While fibulin-2 is cleaved by a variety of proteases and MMP family members, fibulin-1 shows a more limited susceptibility, in that, among the proteases tested, only leukocyte elastase and matrilysin were active against the protein. Imai *et al.* (1995b) recently showed that matrilysin digests vitronectin from 8- to 44-fold more efficiently than either gelatinase A, gelatinase B, or stromelysin-1. Interestingly, it appears that human matrilysin, but not the rat homologue, degrades insoluble elastin (Woessner and Taplin, 1988; Murphy *et al.*, 1991; Imai *et al.*, 1995a), which is one of the few indications that there may be subtle differences between the rodent and human enzymes.

Several other ECM molecules are cleaved by matrilysin, including the proteoglycan aggrecan and its link protein, versican, and tenascin. In fact, bovine nasal cartilage proteoglycan (now termed aggrecan) was the first substrate that matrilysin was shown to degrade (Sellers and

Woessner, 1980). In comparison to other MMPs, matrilysin most extensively degrades the components of cartilage proteoglycan aggregates, which include the link protein that stabilizes the interaction between aggrecan and hyaluronic acid in these aggregates (Nguyen *et al.*, 1993). Like entactin/nidogen, aggrecan is composed of globular domains separated by a protease-sensitive region. Stromelysin-1, the gelatinases, collagenase-1 and -2, and matrilysin all cleave at the same bond in this interglobular region; however, matrilysin and the collagenases recognize an additional site in proximity to the second globular domain (Fosang *et al.*, 1992, 1993). Matrilysin also degrades the proteoglycan versican more efficiently than equimolar concentrations of collagenase-1, gelatinase B, or stromelysin-1 (Halpert *et al.*, 1996), making it one of the most potent proteoglycanases among the MMPs. Finally, the 200-kD isoform of tenascin-C, which is generated by alternative splicing of seven fibronectin-like type III repeats, is susceptible to degradation by matrilysin, but not other MMPs; in contrast, the large form (300 kD), which includes those repeats, is digested by matrilysin, gelatinase A, and collagenase-1 within the type III repeats (Siri *et al.*, 1995). From all these studies, we can infer that the carboxyl-terminal hemopexin-like domain absent in matrilysin does not play a role in determining bond recognition in all substrates, because matrilysin and other MMPs cleave some of the same sites. However, it is clear that differential cleavage of molecules by MMPs, even those grouped by similar substrate specificity, is possible, and may be mediated by residues adjacent to the cleavage site and/or secondary structure at that site in the substrate.

From the preceding discussion, it appears that matrilysin targets components of basement membranes and has the potential to degrade proteins associated with remodeling or provisional matrices. As discussed later, since expression of matrilysin is observed in a variety of epithelial tissue and epithelial-derived tumor cells, it is not too surprising that this metalloenzyme has such an affinity for basement membrane proteins. These findings have led us and others to speculate that matrilysin may have a role in epithelial cell homeostasis, and, when aberrantly expressed in neoplastic lesions, may facilitate tissue breakdown associated with the growth and spread of tumor cells. Do the cleavage activities defined by *in vitro* biochemical assays occur *in vivo*, and are they biologically relevant? Although the answers to these questions are actively being pursued, a few studies have attempted to address some of these issues. For example, von Bredow and co-workers (1995) showed that recombinant matrilysin added to fibroblast cultures degrades fibronectin fibrils assembled by the cells. Several fragments were generated, two of which were derived from the cell-binding region.

Similarly, Fukai *et al.* (1995) found that matrilysin liberated cell-binding activity from fibronectin, whereas stromelysin-1, collagenase-1, and gelatinase A tended to produce amino- and carboxyl-terminal fragments. In addition, cleavage of entactin/nidogen by matrilysin generates biologically active peptides that promote neutrophil chemotaxis and phagocytosis (Gresham *et al.*, 1996). These experiments further support the idea that matrilysin-mediated degradation of ECM components has physiological significance *in vivo*.

B. ProMMPs

In addition to its ability to degrade ECM components, matrilysin may have an indirect role in matrix remodeling by activating the latent forms of other MMPs. Quantin *et al.* (1989) found that treatment of procollagenase-1 with activated matrilysin and APMA increased the level of collagenase-1 activity five-fold over that observed using APMA alone. In contrast, stromelysin-1 elicited a higher level of activity at a lower concentration of enzyme, indicating that it is a more efficient collagenase-1 activator than matrilysin, although both enzymes generate the same amino terminus (Sang *et al.*, 1996). Neither human nor rat matrilysin appear to cleave procollagenase-3 (Abramson *et al.*, 1995), which is the only identified interstitial collagenase present in rodents (see Chapter 2). Matrilysin excises the propeptide directly from the latent form of gelatinase A in the absence of any autoproteolysis (Crabbe *et al.*, 1994); cleavage of progelatinase A by both matrilysin and collagenase-1 has been confirmed by Sang *et al.* (1996). Another report indicates that, in a manner similar to that of stromelysin-1 and trypsin, both matrilysin and collagenase-1 generate an active 65-kDa form of neutrophil gelatinase B (Sang *et al.*, 1995). Thus, in situations where these metalloenzymes are coexpressed, matrilysin may act as a physiological activator of other MMP family members.

C. Nonmatrix Proteins

In vitro, matrilysin cleaves several nonmatrix proteins such as casein, insulin, transferrin (Woessner and Taplin, 1988), α_1 -antitrypsin/ α_1 -proteinase inhibitor (Sires *et al.*, 1994; Zhang *et al.*, 1994), pro-uPA and uPA (Marcotte *et al.*, 1992), proTNF- α (Gearing *et al.*, 1994), and myelin basic protein (Chandler *et al.*, 1995). Cleavage of some of these proteins by matrilysin may be involved in modulating cellular behavior. For example, matrilysin was identified as the proteinase responsible for converting the high-molecular-weight form of both pro-uPA and active uPA into two fragments (Marcotte *et al.*, 1992), one of which makes up the receptor-binding domain and the other the serine protein-

ase region. The receptor-binding fragment has been shown to have growth factor-like activities for certain cell types (e.g., see Anichini *et al.*, 1994). Similarly, Gearing *et al.* (1994) showed that purified matrilysin, along with stromelysin-1 and collagenase-1, is particularly adept at processing a recombinant, truncated form of the TNF- α precursor to its mature form. Normally, proteolytic processing of the precursor results in release of the membrane-bound precursor as the active cytokine, and this processing is prevented by synthetic MMP inhibitors (Gearing *et al.*, 1994). Although a cell-surface member of the ADAM (a disintegrin and metalloproteinase) or adamalysin family of metalloproteinases has been identified as the primary TNF- α convertase (Black *et al.*, 1996, 1997; Moss *et al.*, 1997), matrix proteinases may still be involved at some stage in the processing in specific tissue environments. Several investigators have shown that activated matrilysin may regulate the activity of the serine proteinase leukocyte elastase through its ability to cleave the major physiological inhibitor of this enzyme, α_1 -antitrypsin/ α_1 -proteinase inhibitor (Sires *et al.*, 1994; Zhang *et al.*, 1994). Matrilysin was found to be from 30- to 180-fold more effective at digesting α_1 -antitrypsin than other MMPs tested (Sires *et al.*, 1994). Therefore, as an elastase itself and as an inactivator of elastase inhibitors, matrilysin may act to enhance elastin degradation. Matrilysin has been proposed to be a general serpinase, or inactivator of serine proteinase inhibitors, that could play a role in inflammation and tissue damage (Zhang *et al.*, 1994).

D. Cleavage Specificity

The composition of the peptide bonds recognized by matrilysin in the substrates described earlier follows the general trend first noted by Woessner and Taplin (1988) for the B chain of insulin, which has cleavage sites at Ala¹⁴-Leu¹⁵ and Tyr¹⁶-Leu¹⁷. Matrilysin shows a preference for a Leu residue in the P₁' position and generally a hydrophobic residue in the P₁' position. Accordingly, in pro-uPA and α_1 -antitrypsin, matrilysin cleaves at a single site containing Leu in the P₁' position. It also recognizes Leu-containing bonds in aggrecan and cartilage link protein. To dissect further the bond specificity of matrilysin, several groups have analyzed its cleavage efficiency against synthetic peptide substrates. Using a series of octapeptides, Netzel-Arnett *et al.* (1993) found that matrilysin exhibited a particular affinity for sites with Leu at P₁' and Met at P₃, and, unlike the gelatinases, was able to accommodate residues with bulkier side groups than that of Gly or Ala in the P₁ position. Of the peptides tested, the ones showing the highest activity as compared to the reference peptide Gly-Pro-Gln-Gly ↓ Ile-Ala-

Gly-Gln (from P_4 to P_4') were Gly-Pro-Gln-**Ala** ↓ Ile-Ala-Gly-Gln > Gly-Pro-Gln-Gly ↓ Ile-Ala-**Met**-Gln > Gly-Pro-**Leu**-Gly ↓ Ile-Ala-Gly-Gln > Gly-Pro-**Met**-Gly ↓ Ile-Ala-Gly-Gln (where the altered residues are shown in bold). To identify potentially new cleavage sites, as well as confirm the site preferences observed previously, Smith and co-workers (1995) constructed a phage display library to test with activated recombinant matrilysin. They obtained the highest k_{cat}/K_M ratio with the hexapeptide Pro-Leu-Glu ↓ Leu-Arg-Ala (from P_3 to P_3'). From this assay, they could clearly distinguish between the preference of stromelysin-1 for Phe and Met at positions P_2 and P_1' , respectively, and the preference of matrilysin for Leu at both of these positions, despite the similarity in substrate recognition normally shared by these enzymes. Because matrilysin and stromelysin-1 differ at residues 214 and 215 in the substrate-binding region (S_1'), Welch *et al.* (1996) introduced mutations into this region to attempt to determine the basis for the P_1' preferences. When either residue 214 or both residues 214 and 215 in matrilysin were replaced by amino acids present in stromelysin-1, the mutants showed P_1' preferences characteristic of stromelysin-1. Precise molecular studies such as those described here could lead to the design of specific synthetic inhibitors potentially useful in therapeutic intervention in cancer and other diseases.

E. Inhibition of Activity

Matrilysin and other MMPs are inhibited by metal-chelating agents such as EDTA and 1, 10-phenanthroline, which are most useful for *in vitro* biochemical studies. A number of synthetic MMP inhibitors that act as substrate analogues have also been developed and are active against matrilysin (see Chapter 11). Serum inhibitors of matrilysin include the α -macroglobulins and α_1 -inhibitor₃ (Zhu and Woessner, 1991). In addition, there are tissue inhibitors of MMPs (TIMPs) of which TIMP-1 and TIMP-2 are known to form noncovalent 1 : 1 complexes with the active form of matrilysin at the catalytic site. However, the mechanism by which binding of TIMPs inhibits MMP activity is still not entirely clear. Using high-resolution gel filtration chromatography, Baragi *et al.* (1994) compared the ability of matrilysin, truncated stromelysin-1, and full-length stromelysin-1 to bind TIMP-1. While each of these molecules was able to form a complex with TIMP-1 that was resistant to separation by gel filtration, both matrilysin and truncated stromelysin-1 showed a reduced ability to bind TIMP-1 when competing with full-length stromelysin-1. These data indicate that the carboxyl-terminal domain absent in matrilysin contributes to the affin-

ity of MMPs for their physiological inhibitors and lead to the hypothesis that matrilysin may be more resistant to inhibition than other MMPs. Note that other MMPs, metalloelastase in particular (see Chapter 7), can undergo carboxyl-terminal processing to remove the hemopexin-like domain at the hinge region.

V. EXPRESSION IN CANCER AND OTHER DISEASES

Human matrilysin was first cloned from a human cDNA library prepared from a pool of primary and metastatic tumor RNAs. In fact, until a few years ago, matrilysin expression in human tissue had been associated *primarily* with neoplastic lesions in a variety of organs, suggesting that this metalloenzyme plays a role in tumor invasion and metastasis. Matrilysin has been detected in lesions of the breast (Basset *et al.*, 1990; Wolf *et al.*, 1993; Heppner *et al.*, 1996), stomach (McDonnell *et al.*, 1991; Honda *et al.*, 1996), colon (McDonnell *et al.*, 1991; Yoshimoto *et al.*, 1993; Newell *et al.*, 1994; Yamamoto *et al.*, 1994; Mori *et al.*, 1995; Ishikawa *et al.*, 1996), prostate (Pajouh *et al.*, 1991; Knox *et al.*, 1996b), upper aerodigestive tract (oral cavity, oropharynx, hypopharynx, and endolarynx) and lung (Muller *et al.*, 1991; Bolon *et al.*, 1996), and skin (Karelina *et al.*, 1994). *In situ* hybridization and immunohistochemistry localized expression primarily to the tumor cells in both benign and malignant neoplasms; other MMP family members usually originate in inflammatory cells or are induced in the surrounding stroma, although epithelial expression of stromal MMPs in advanced lesions has been observed (e.g., see Wright *et al.*, 1994; reviewed in MacDougall and Matrisian, 1995; Powell and Matrisian, 1996). In addition, Wang *et al.* (1995) detected matrilysin mRNA in neoplastic cells of several osteosarcoma subtypes, and protein was found in cholangiocellular carcinoma of the liver (Lichtinghagen *et al.*, 1995). Matrilysin is also expressed in glioma specimens and several cell lines established from these lesions, which are characteristically highly destructive to tissues (Nakano *et al.*, 1993, 1995). Varying levels of the mRNA have been detected in some cell lines derived from colon and breast adenocarcinomas (Gaire *et al.*, 1994; and L. M. M., unpublished observations), and the protein was isolated from a rectal carcinoma-derived cell line (Miyazaki *et al.*, 1991). Another group used casein zymography to correlate the levels of enzyme with tumor stage of colorectal lesions, and found that while matrilysin was readily detectable in adenomatous and cancerous tissue, it was not present in hyperplastic polyps or normal colon tissue (Itoh *et al.*, 1996). Together, these observations clearly show that matrilysin expression is a characteristic feature of many cancerous lesions, particularly those of epithelial and glandular epithe-

lial origin. In addition, its appearance in adenomas suggests that it may function early in tumor development.

Several groups have used gene transfer approaches to study the effect of matrilysin overexpression and underexpression on tumor cell behavior. One of the first definitive experiments was carried out by Powell *et al.* (1993), who showed that when the nonmetastatic prostate tumor cell line DU-145 was transfected with human matrilysin cDNA, the cells were significantly more invasive in an *in vivo* model of cell invasion. This finding suggested that matrilysin may be involved in basement membrane degradation as an initial step in tumor cell extravasation. In support of this hypothesis, Imai and co-workers found that introduction of sense or antisense matrilysin constructs into BM314 colon cancer cells resulted in an increase or a decrease, respectively, in the ability of the cells to invade an artificial membrane *in vitro* (Yamamoto *et al.*, 1995b; Itoh *et al.*, 1996). In contrast, overexpression of a similar cDNA construct in the SW480 colon tumor cell line (which, like the DU-145 cells, does not endogenously express matrilysin) did not reproducibly stimulate invasion when the cells were injected into the cecum of nude mice (Witty *et al.*, 1994); instead, an increase in the tumorigenicity of the cells was observed. This finding was confirmed by the observation that orthotopic injection of antisense SW620 clones led to a decrease both in tumorigenicity and incidence of metastasis (Witty *et al.*, 1994). Unlike the Imai studies, we and others saw no statistically significant difference between matrilysin-transfected and control cells in their ability to traverse an artificial basement membrane. Even when a cDNA containing an activating mutation in sequence encoding the pro-domain was introduced into the SW480 cells, only one clone of five showed an increase in invasion (Witty *et al.*, 1994), indicating that matrilysin alone is not always sufficient for cell migration through a matrix barrier. It is possible that the differences in cellular responses observed in these studies may reflect differences in cell type and cell line, in the systems used for measuring cell invasion, and/or in the other MMPs that may be elaborated by these cells. Multiple members of the MMP family probably cooperate to degrade basement membrane components to enhance the invasive process. Degradation of the matrix may, in turn, increase expression of specific enzymes by a positive feedback mechanism. For example, Yamamoto *et al.* (1994) showed that the level of matrilysin mRNA was up-regulated in WiDr colon carcinoma cells plated on immobilized FN fragments containing the cell-binding domain. As mentioned earlier, a fibroblast-derived factor stimulates expression of matrilysin in a human squamous cell line (Borchers *et al.*, 1994), suggesting that its production in epithelial cells is not completely independent of the stromal component in all cases.

The differences noted earlier, along with the observation that matrilysin is expressed early in tumorigenesis, also suggest that matrilysin has dual roles in tumor progression, depending on the temporal and spatial pattern of its appearance. In addition to the effects on tumorigenicity observed *in vivo*, expression of matrilysin in early adenomatous lesions is indicative of its involvement in processes such as cell growth and proliferation. We envision that the induction of matrilysin, perhaps as a result of oncogenic mutations and/or alterations in the matrix environment, may favor the growth and expansion of premalignant cells under the proper conditions by an as yet unknown mechanism. Then, as development of the tumor progresses, sustained production of matrilysin, probably in conjunction with stromal and leukocyte-derived MMPs, could lead to tissue degradation, cell invasion, and metastasis to distant sites via classical matrix-degrading activities. To begin to test these ideas, and to attempt to define the mechanism by which matrilysin could promote tumor formation, our laboratory has generated several mouse models in which levels of matrilysin have been genetically manipulated.

Mice lacking matrilysin were created using standard methods of gene ablation in embryonic stem cells (Wilson *et al.*, 1997). Pups homozygous for the targeted allele (*Mmp7^{Wu}*) were produced in the expected Mendelian ratio from heterozygote matings, and, under conditions of conventional barrier housing, these mice have shown no obvious defects throughout their life span. Because matrilysin is constitutively expressed in the glandular epithelium of the reproductive tract of both males and females (see later discussion), we expected there might be deleterious effects on fertility, but none were noted (Rudolph-Owen *et al.*, 1997a). To assess the role of matrilysin in the development of adenomatous lesions, we elected to focus on intestinal tumors that spontaneously form in mice with the *Apc^{Min}* mutation. We demonstrated by *in situ* hybridization that the majority of these tumors (90%) express high levels of matrilysin (Wilson *et al.*, 1997). Interestingly, the protein was predominantly localized to the apical face of these cells and to the lumen of the dysplastic glands, rather than to the basement membrane (Fig. 2b). Matrilysin-deficient mice were bred to these mice and examined for tumors at an age (4 months) at which lesions are clearly evident in wild-type (*Mmp7^{+/+}*) *Min* animals. On the matrilysin-null background, we found that the number of tumors was significantly reduced (by 58%), and that the tumors were on average smaller in diameter than those in control *Min* mice (Wilson *et al.*, 1997). Matrilysin knock-out/*Min* animals analyzed at 6 months, an age to which most wild-type *Min* animals do not survive, exhibited many tumors, indicating that a deficiency in matrilysin results in a *delay* in tumor develop-

ment. Although the mechanism by which matrilysin promotes tumor formation in the gastrointestinal tract has yet to be elucidated, we speculate that it may affect the proliferation of transformed epithelial cells, perhaps by modulating growth factor accessibility and/or production of stimulatory degradative fragments of ECM molecules.

Complementary experiments in which matrilysin has been overexpressed *in vivo* have solidified the concept of this metalloenzyme as a critical regulator of the tumorigenic phenotype. Transgenic mice in which a human matrilysin mini-gene was targeted to the mammary gland epithelium via the mouse mammary tumor virus promoter were generated recently (Rudolph-Owen *et al.*, 1997b); a significant number of multiparous transgenic females showed focal areas of epithelial hyperplasia, or hyperplastic alveolar nodules (HANs), which are believed to be precursors of mammary carcinomas. Formation of these HANs may be related to the precocious epithelial cell differentiation that was observed in these animals, because virgin mammary glands were found to produce milk proteins prematurely (Rudolph-Owen *et al.*, 1997b). Furthermore, when these transgenic mice were bred to animals expressing the oncogene *neu* under the same promoter, the resulting progeny developed mammary tumors 13 weeks earlier, and at a higher frequency (100% versus 80%), than the *neu* controls. However, no obvious differences were observed between these two groups in the growth rate of *neu*-induced tumors or in the number of lung metastases (Rudolph-Owen and L.M.M., manuscript in preparation). Therefore, these results point to an effect of matrilysin on *development* of the mammary tumors, somewhat analogous to its effect on the formation of intestinal tumors. *Neu*-induced tumors alone do not express matrilysin; indeed, Goto *et al.* (1995) detected matrilysin in pregnancy-dependent mammary tumors of mice, but not in hormone-independent lesions. In these studies, matrilysin expression was found to be highest during the initiation and regression stages of these tumors.

Recent studies have shown that the expression of matrilysin is also a feature of diseases other than cancer, in particular disorders characterized by active tissue remodeling. Saarialho-Kere and co-workers (1996) examined samples of gastric ulcers, Crohn's disease, and ulcerative colitis for matrilysin expression by *in situ* hybridization and immunohistochemistry. While another group was unable to detect matrilysin expression in *mild* cases of ulcerative colitis (Yoshimoto *et al.*, 1993; Yamamoto *et al.*, 1994; Itoh *et al.*, 1996), Saarialho-Kere *et al.* (1996) found matrilysin localized to the mucosal epithelium bordering the ulcer in the majority of lesions, and signal for this metalloenzyme was not evident in the stroma or inflammatory cells. In addition, immunostaining for laminin and fibronectin in the basement membrane be-

neath the matrilysin-positive cells was weak compared to distal sites showing no matrilysin expression, suggesting that the enzyme may have degraded these glycoproteins. Furthermore, absence of a signal for the proliferation marker Ki-67 led these investigators to hypothesize that matrilysin expression is associated with cell migration, rather than proliferation, in this type of lesion. Support for this hypothesis was obtained in a recent study by Parks *et al.* (1997), who showed expression of matrilysin in migrating airway epithelial cells in an *ex vivo* model of tracheal wound repair. They also found high levels of matrilysin mRNA and protein in bronchiolar cells from patients with cystic fibrosis (Fig. 3), suggesting that this MMP in particular is up-regulated in response to epithelial damage and that it plays a role in reparative processes in the lung. In general, it appears that matrilysin expression is limited to glandular or luminal epithelium in tissue repair, and is not expressed in the mature epidermis or in skin wounds or lesions (Saarialho-Kere *et al.*, 1993; Vaalamo *et al.*, 1996). However, some cutaneous and lung inflammatory diseases, such as cystic fibrosis, do show matrilysin production in occasional blood vessel-associated monocytes and freshly extravasated tissue macrophages (Busiek *et al.*, 1995). In atherosclerotic plaques, matrilysin, along with metalloelastase, is expressed in lipid-laden macrophages only at junctions between the lipid core and fibrous cap (Halpert *et al.*, 1996). This precise and confined localization implicates matrilysin activity in the structural instability of these lesions. The finding that versican, a blood vessel chondroitin sulfate proteoglycan, is abundant in these plaques and is degraded more readily by matrilysin than other MMPs tested, suggests one potential mechanism for plaque rupture (Halpert *et al.*, 1996). Interestingly, other MMPs, namely, collagenase-1, stromelysin-1, and gelatinase B, are also expressed by lipid-laden macrophages in atherosclerotic plaques, but *within* the cellular areas of the fibrous cap and shoulder (Henney *et al.*, 1991; Galis *et al.*, 1994; Brown *et al.*, 1995; Nikkari *et al.*, 1995). Thus, subpopulations of macrophages can be defined by their location and profile of MMP expression, suggesting distinct regulatory mechanisms for and functions of the family members. Matrilysin expression is associated with several other pathologies: The protein was detected in cells of the synovial lining in a single case of atypical rheumatoid arthritis characterized by heavy infiltration of inflammatory cells (Hembry *et al.*, 1995). Also, Vettriano and co-workers (1996) found that matrilysin, which is normally expressed in cytotrophoblastic cells during pregnancy, is produced at increased levels and by several cell types in the placenta in preeclampsia.

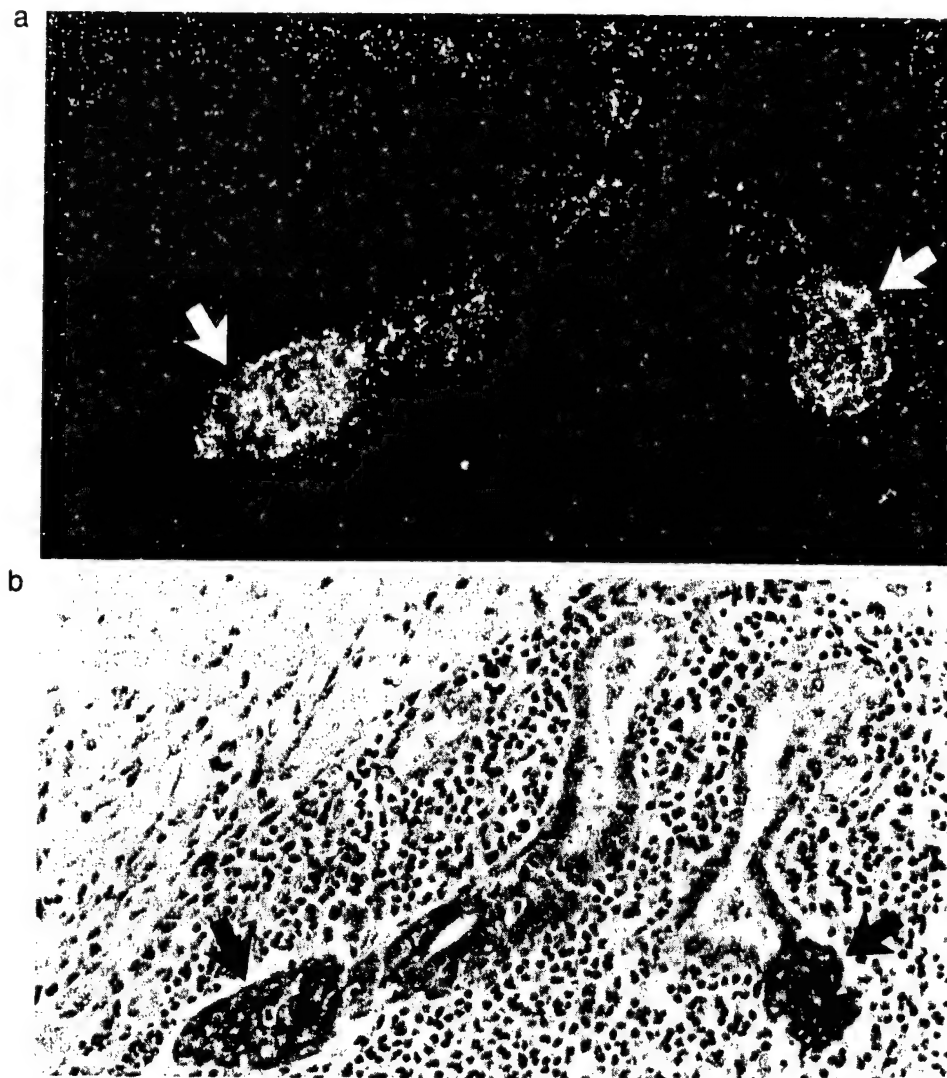


FIG. 3. Colocalization of matrilysin mRNA and protein within damaged epithelium. Serial sections of airway tissue from a patient with cystic fibrosis were processed for (a) *in situ* hybridization and (b) immunohistochemistry using probes specific for human matrilysin as described in Saarialho-Kere *et al.*, 1995. The arrows point to glandular structures positive for matrilysin by both assays. (Photomicrographs courtesy of W. C. Parks.)

VI. ROLE IN NORMAL TISSUE REMODELING AND HOMEOSTASIS

Early analysis of matrilysin expression in human neoplasms revealed that the mRNA appears in the surrounding normal tissue in a few limited cases. For example, while matrilysin was not present in normal

gastric or colonic tissue (McDonnell *et al.*, 1991), the message was detected at low levels in the bronchial mucosa (Muller *et al.*, 1991) and in 3 of 11 normal prostate samples (Pajouh *et al.*, 1991) by Northern hybridization. However, this type of analysis precluded identification of the cell type responsible for matrilysin synthesis. Because it had been shown earlier that matrilysin is expressed in the early involuting uterus of the rat (Woessner and Taplin, 1988; Quantin *et al.*, 1989), Rodgers *et al.* (1993) used a combination of Northern blotting, *in situ* hybridization, and immunohistochemistry to examine the expression of matrilysin in the human endometrium, a tissue that undergoes rapid remodeling during the normal menstrual cycle. These investigators found that matrilysin mRNA was expressed in the proliferative, late secretory, and menstrual phases of the cycle. Furthermore, both the mRNA and protein localized to the epithelium of endometrial glands. A subsequent investigation (Rodgers *et al.*, 1994) showed that other MMPs are expressed in a cycle-specific pattern in the endometrium as well, but the mRNAs encoding these enzymes localized to the stroma rather than the epithelium. This work probably provided the first indication that the cell-type specificity of matrilysin expression that was observed in cancerous lesions would be recapitulated in normal tissue.

Several other studies have since confirmed and extended these observations to other organs and tissues in humans. For example, matrilysin protein was localized to epidermal cells in the developing fetal skin, where it was prominently expressed in cells invading the mesenchyme in budding hair follicles and sweat glands (Karelina *et al.*, 1994). In adult skin, the protein was detected in the outer root sheath of the hair follicles and in the secretory cells of the sweat glands (Karelina *et al.*, 1994). In fact, Saarialho-Kere *et al.* (1995) showed that matrilysin is produced by most exocrine glands in the body, including the mammary and parotid glands, the pancreas, liver, prostate, and peribronchial glands of the lung. Wolf and co-workers (1993) demonstrated by *in situ* hybridization that matrilysin is expressed in non-neoplastic as well as neoplastic cells in human breast tissue. In addition, Honda *et al.* (1996) observed low levels of matrilysin protein in normal gastric epithelial cells. Both matrilysin mRNA and protein were localized to ductal and secretory epithelium in the organs mentioned and were frequently found to colocalize, as depicted in a representative section of a damaged airway in Fig. 3. However, in some cases, such as the eccrine sweat glands, the mRNA was observed in ductal epithelial cells while protein was localized to secretory areas of the glands (Saarialho-Kere *et al.*, 1995). These studies also indicated that the protein is secreted in the luminal direction, since staining was not observed in

the underlying stroma. This interpretation was strengthened recently by experiments showing that alveolar type II cells secrete matrilysin from their apical surface when cultured *in vitro* (Parks *et al.*, 1997). Taken as a whole, these results led these investigators to propose that matrilysin participates in the normal function of glands by maintaining the patentability of the glandular lumen (acting as an "enzymatic pipe cleaner"). The constitutive pattern of expression does indicate that matrilysin functions in a capacity that is not related merely to a matrix remodeling process. This idea is reinforced by the pattern of matrilysin expression observed in rodent tissue, as described later. Matrilysin is also expressed by a few other cell types in humans. For example, matrilysin is produced by cultured fetal kidney cells, cytokine-stimulated glomerular mesangial cells, and in diseased kidney (Marcotte *et al.*, 1992; Marti *et al.*, 1992). Matrilysin is synthesized by promonocytes extracted from bone marrow and by peripheral blood monocytes following brief culture *in vitro* (Busiek *et al.*, 1992). As mentioned previously, tissue macrophages positive for matrilysin are seen in atherosclerosis and inflamed lung (Busiek *et al.*, 1995; Halpert *et al.*, 1996). However, it is not produced by fully differentiated pulmonary alveolar macrophages (Busiek *et al.*, 1992). Lastly, a recent report indicates that matrilysin protein is produced in cytotrophoblasts and syncytioblasts during the first trimester of pregnancy, and appears in intermediate trophoblasts and decidual cells during all stages (Vetraino *et al.*, 1996). Overall, however, matrilysin expression is restricted to glandular epithelium, with the repertoire of cell types producing the enzyme expanding to a very limited degree in tissue remodeling disorders.

The first study to show that normal tissue is capable of producing high levels of matrilysin under nonpathological conditions was that of Sellers and Woessner (1980) in their work on uterine involution in the rat. Analyzing matrilysin expression and function in rodents has been of great interest in the last few years due to the experimental and genetic malleability of these organisms. Based on Woessner's work, we examined matrilysin expression in the mouse postpartum uterus, from which the cDNA was cloned, as well as in the pregnant and cycling uterus. We found that the mRNA is expressed at high levels late in gestation, with levels increasing immediately after birth and then decreasing until about day 4.5 postpartum (Wilson *et al.*, 1995), in agreement with Woessner's results and more recently those of Wolf *et al.* (1996) in the rat. Matrilysin is also produced during several stages of the estrous cycle in both mice (Rudolph-Owen *et al.*, 1997a) and rats (Wolf *et al.*, 1996; Woessner, 1996). In addition, Wolf *et al.* noted expression of matrilysin in the rat cervix in a temporal pattern similar to

that of the uterus, suggesting it may have a role in cervical ripening (Woessner, 1996). In all uterine samples, we found the message localized to epithelial cells lining the lumen of the uterus and associated glandular structures. Using a polyclonal antibody against the carboxyl-terminal segment of matrilysin, we detected protein in epithelial cells of the involuting uterus, and found that it appeared to be secreted *apically* into the lumen (Fig. 2a). This result was in marked contrast to our expectations; we had anticipated that matrilysin, like other MMPs expressed in the postpartum uterus, would have a role in tissue degradation and remodeling during the process of involution. One role that we had postulated for matrilysin in this tissue is that it might serve to activate procollagenase-3, a stromal MMP critical for clearance of excess collagen from the uterus. However, because procollagenase-3 is able to cleave itself efficiently at the site that, in procollagenase-1, is recognized by matrilysin, it has been difficult to determine whether matrilysin is truly capable of catalyzing this reaction (Abramson *et al.*, 1995). In addition, matrilysin-null animals appear to undergo involution normally (Rudolph-Owen *et al.*, 1997a), although a rigorous time course on the kinetics of tissue resorption has not been done. Secretion of matrilysin protein from the apical side of these cells suggests a role for the enzyme either on the cell surface or in the lumen.

In the male reproductive tract, matrilysin transcripts were localized to epithelial cells of the efferent ducts, and, in mature animals, to cells of the initial segment and cauda of the epididymis (Wilson *et al.*, 1995); the mRNA has also been detected in the seminal vesicles (W.C. Powell and L.M.M., unpublished observations). This localization pattern led us to speculate that matrilysin plays a role in the maturation and progression of sperm through the extratesticular ducts. While this possibility has not been entirely ruled out, the observation that matrilysin-null animals show no overt deficiencies in either fertility (ability to reproduce) or fecundity (number of offspring) indicates that matrilysin function in these ducts is either dispensable, is replaced by another protease, or has not been revealed by the experiments carried out so far. Attempts to demonstrate alterations in the processing of fertilins, members of the ADAMs which are differentially expressed in the testis and associated ducts, in these mice have proven futile (C.L.W., R. Yuan, D. Myles, and L.M.M., unpublished observations). We have also examined the repertoire of proteins produced in both the tissue and luminal fluid of the epididymis, as well as the sperm, and have not discovered any significant differences in the profile of processed proteins (C.L.W. and L.M.M., unpublished observations). In addition to the constitutive level of matrilysin produced by some extratesticular ducts, Powell *et al.* (1996) showed that matrilysin is induced in the rat

ventral prostate as it undergoes resorption in response to androgen withdrawal. Again, expression was restricted to the epithelial cells of the involuting gland. As uPA is also expressed during prostate involution, these workers propose that both metallo- and serine proteases play a role in remodeling of the prostate tissue architecture. Matrilysin knock-out mice will be extremely useful for addressing these questions and deciphering the potential interaction between members of the different protease families.

Another organ in which matrilysin is constitutively expressed at high levels in the mouse is the small intestine (Wilson *et al.*, 1995). Using *in situ* hybridization, we observed that the mRNA was localized only to the Paneth cells at the base of crypts, and was not present in other epithelial cell lineages in the small intestine (Wilson *et al.*, 1995). Although message was also detected in the stomach and colon by reverse transcription-polymerase chain reaction (RT-PCR), we were unable to localize expression by *in situ* hybridization and concluded that expression in these organs is very low (Wilson *et al.*, 1995). Along with other Paneth cell markers, namely, cryptdin-4, -5, and -6, matrilysin mRNA was detected in the P1 (postnatal day 1) small intestine by RT-PCR (Darmoul *et al.*, 1997), suggesting that the undifferentiated intervillus epithelium is capable of producing these proteins prior to crypt formation. The precise role of the Paneth cell has been somewhat enigmatic, but, given that proteins such as lysozyme and the cryptdin peptides (crypt defensins) are synthesized by these cells, they likely act as specialized defense cells in the gastrointestinal system. Does matrilysin expression contribute to the function(s) of the Paneth cell *in vivo*? And, more important, does matrilysin's activity in these cells relate to its role in other epithelial cells? Again, with mice lacking matrilysin available, we can begin to delve into these issues.

As alluded to previously, rodents and humans show striking similarities in the pattern of matrilysin expression in normal organs and tissues. For example, the mRNA has been detected in ducts of the breast, the bile duct, and peribronchial glands of the lung in humans (Saarialho-Kere *et al.*, 1995); similarly, we have found that the mammary gland (cycling, lactating, and involuting) and lung in mice are positive for matrilysin by RT-PCR, and transcripts have been localized to an extrahepatic branch of the bile duct in these animals (Wilson *et al.*, 1995). However, there are some differences between the species in the organs that express matrilysin. While matrilysin was found in ducts of the parotid glands and pancreas in humans, it was absent from these organs in the mouse, at least at the detection level of *in situ* hybridization. In contrast to the mouse, matrilysin does not appear to be produced by Paneth cells in humans (Saarialho-Kere *et al.*, 1996;

and personal communication); however, it can be detected in the decidua (Vettraino *et al.*, 1996), whereas this tissue is negative in mice (Wilson *et al.*, 1995). Some of these differences may reflect the techniques used for visualizing expression, and some may represent true disparities between the species. There are certainly species-specific events associated with tissue remodeling; for example, in humans and other primates, uterine tissue is restructured and the endometrial lining ultimately shed during the menstrual cycle, whereas in the mouse, the uterus undergoes an extremely rapid estrous cycle characterized by alternating phases of active cell growth, degeneration, and quiescence. Tissue variabilities such as these are probably important in dictating the repertoire and activity of molecules expressed *in vivo*. Equally possible is that other molecule(s) may substitute for matrilysin in some instances, as the pathways controlling expression of this protease may have evolved differently between rodents and humans. The tissue microenvironment and architecture differs in some organs of these mammals, as well, as has been observed in the skin (Cohen and Mast, 1990), and these differences probably govern the specific molecules involved in remodeling of those tissues. Despite these caveats, the overwhelming similarities in organ structure and function between the species justify using genetically altered mice as suitable model systems for human physiology and disease.

VII. SUMMARY AND CONCLUDING REMARKS

Among the MMP family members characterized thus far, matrilysin is unique in that the gene encodes only the minimal domains required for activity. Although the hemopexin-like domain present in other MMPs can be removed by proteolysis *in vitro*, it is not clear if and when this processing occurs *in vivo*, suggesting that the structure of matrilysin is most likely functionally distinctive. Matrilysin has traditionally been grouped with the stromelysins based on its ability to cleave a wide range of ECM glycoproteins, although it shows a greater similarity to the collagenase-1 catalytic domain with regard to the structure of its substrate-binding pocket. Of the stromelysins, matrilysin has primarily been compared with stromelysin-1 as to substrate bond specificity and rate of catalysis. For several ECM substrates, activated matrilysin was found to be a more efficient enzyme than stromelysin-1, at least *in vitro*, and often cleaved at sites distinct from those recognized by stromelysin-1. This observation, along with the finding that in both normal and neoplastic tissue, matrilysin is characteristically of epithelial rather than stromal origin, suggests that it may function in a manner unlike that of other MMPs. Although experi-

ments *in vitro* show that matrilysin is able to cleave matrix components, the finding that the protein is primarily localized to the apical face or lumen of glandular epithelium implicates this MMP in extracellular activities unrelated to ECM reorganization. Although it may degrade matrix structures when secreted basally, particularly in actively remodeling tissue, the luminal secretion of matrilysin suggests that this molecule participates in homeostatic processes such as host defense, cell proliferation, and protein turnover (Fig. 4). Because mice deficient in this MMP are viable, matrilysin is not absolutely essential for these processes in normal embryonic and postnatal development. Furthermore, under conditions of conventional housing, matrilysin-null mice have exhibited no overt phenotypic alterations. However, growth of genetically induced adenomatous lesions in the intestinal tract does appear to be delayed in the knock-out mice as compared to wild type animals, indicating there is an association between matrilysin expression and growth control *in vivo*. This finding also attests to the need for the appropriate stimulus or experimental test to reveal the potentially subtle differences that may exist between matrilysin knockout and wild-type mice, especially if we consider that compensatory mechanisms or redundant pathways may be involved.

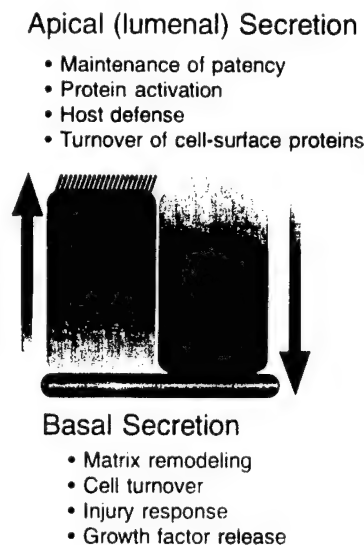


FIG. 4. This schematic depicts the functions that have been proposed for matrilysin based on its vectorial secretion in polarized epithelial cells. Constitutive expression of matrilysin in a variety of glandular epithelia and its delivery to the apical cell surface and lumen suggest that it acts as a sentinel molecule in maintaining tissue homeostasis and properly functioning glands. In contrast, induction and basal secretion of the protease appear to be associated with dynamic processes of cell activation and tissue remodeling.

Because matrilysin is constitutively expressed by a variety of glandular epithelia, we propose that it is acting as a sentinel molecule in these cells, maintaining the gland or duct in a state poised for rapid response when the critical signals are received. The challenge for us is to identify those cellular responses, the signals that initiate them, and the precise role that matrilysin plays in them. As part of this challenge, it will be vital to determine the substrates that are recognized and cleaved by matrilysin *in vivo*, particularly since the emerging data indicate that activated matrilysin may be involved in processing of some protein precursors. Does matrilysin nonspecifically cleave luminal contents in the various glandular organs in which it is expressed, or is there some function common to most glandular epithelia that serves as the focus for matrilysin activity? These and other questions can be addressed using genetically defined mice as we have described, as well as other mouse models and the appropriate tissue culture systems.

As with other MMPs, the overexpression of matrilysin has been found to correlate with tumor progression, and it appears to contribute to the ability of tumor cells to extravasate and metastasize, presumably by degradation of the basement membrane. However, the demonstration that matrilysin expression increases the tumorigenic potential of neoplastic colon cells, and our recent finding that mice deficient in matrilysin develop fewer intestinal adenomas than their wild type counterparts, support the idea that this MMP affects the *formation* of tumors, at least in the gastrointestinal tract, in a way that is as yet undefined. Furthermore, the prominent expression of matrilysin in other diseases, such as atherosclerosis, cystic fibrosis, and severe ulcerative colitis, underscores the potential association of this metalloenzyme with growth control, cell migration, and proliferation, in addition to tissue destruction. Perhaps the designation "matrix-degrading enzyme" is too limiting a description for this member of the MMP family. The coming years should hold many more exciting discoveries as details of the way matrilysin functions unfold.

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Matrix Metalloproteinases in Remodeling of the Normal and Neoplastic Mammary Gland

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Alterations in mammary gland structure and function are associated with changes in the expression of members of the matrix metalloproteinase (MMP)³ family of enzymes. In this review, the evidence for a role for specific MMPs in mammary gland development and cellular differentiation, proliferation, and apoptosis is discussed. In addition, MMP expression is altered during the development and progression of preneoplastic and neoplastic breast lesions. The expression of MMP family members in human breast cancer is described, and studies with mouse model systems addressing the role of MMPS in the initiation, growth, invasion, and metastasis of breast neoplasms are reviewed.

KEY WORDS: MMP; matrilysin; stromelysin; gelatinase; collagenase; breast cancer; mammary development.

INTRODUCTION

The mammary gland undergoes substantial structural changes in the adult during ductal development, lactation, and involution. Remodeling of the mammary gland requires both breakdown and re-synthesis of the extracellular matrix components. In pathological conditions such as mammary tumor growth and invasion, disruption of the extracellular matrix also occurs. Degradation and remodeling of matrix proteins in both normal and pathological conditions of the mammary gland can be affected by a variety of enzymatic activities, including the matrix metalloproteinases (MMPs).

The MMPs are a family of zinc-dependent secreted proteases responsible for normal extracellular

matrix remodeling. The MMPs can be grouped loosely by domain structure as well as substrate specificity [reviewed in (1), see Fig. 1]. Most MMPs studied thus far have five domains: A signal sequence or pre domain that directs secretion from the cell, a pro sequence that maintains latency, a catalytic domain containing the critical zinc-binding site, a proline-rich hinge region, and a hemopexin-like domain that is believed to help determine substrate specificity. The collagenases (interstitial collagenase, neutrophil collagenase, and collagenase-3), stromelysin-1, stromelysin-2, and metalloelastase contain these five typical MMP domains. The smallest MMP member, matrilysin, contains the minimal number of domains, the pre, pro and catalytic domains, that are required for secretion and activation but lacks the carboxy-terminal hemopexin-like domain. The gelatinases, gelatinase A and B, contain an additional fibronectin-like region, thought to facilitate binding to their gelatin substrates. Gelatinase B also contains a small insertion in the hinge region that is similar to the $\alpha 2$ chain of type V collagen. The newest members of the MMP family, the membrane-type MMPs (MT-MMPs) are unusual due to the addition of a membrane spanning domain at the carboxy-terminal end which localizes these enzymes to the cell

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³ Abbreviations: matrix metalloproteinase (MMP); murine mammary tumor virus (MMTV); whey acidic protein (WAP); 7,12-dimethylbenzanthracene (DMBA); tissue inhibitor of metalloproteinase (TIMP).

MMP	Number	Structure	Substrates
Matrilysin	MMP-7 EC 3.4.24.23	[Pre] [Pro] [Catalytic Zn⁺⁺]	Proteoglycans, laminin, fibronectin, gelatins, collagen IV, elastin, entactin, tenascin, uPA, Interstitial Collagenase, Gelatinase A, Gelatinase B
Interstitial Collagenase	MMP-1 EC 3.4.24.7	[Pre] [Pro] [Catalytic Zn⁺⁺] [Hemopexin]	Collagens I, II, III, VII, X, gelatins
Neutrophil Collagenase	MMP-8 EC 3.4.24.34	[Pre] [Pro] [Catalytic Zn⁺⁺] [Hemopexin]	Collagens I, II, III
Collagenase-3	MMP-13	[Pre] [Pro] [Catalytic Zn⁺⁺] [Hemopexin]	Collagen I
Stromelysin-1	MMP-3 EC 3.4.24.17	[Pre] [Pro] [Catalytic Zn⁺⁺] [Hemopexin]	Proteoglycans, laminin, fibronectin, collagen III, IV, V, IX, gelatins, Interstitial Collagenase, Neutrophil Collagenase, Gelatinase B
Stromelysin-2	MMP-10 EC 3.4.24.22	[Pre] [Pro] [Catalytic Zn⁺⁺] [Hemopexin]	Proteoglycans, fibronectin, collagen III, IV, V, gelatins, Neutrophil Collagenase
Metalloelastase	MMP-12	[Pre] [Pro] [Catalytic Zn⁺⁺] [Hemopexin]	Elastin, fibronectin, collagen IV
Stromelysin-3	MMP-11	[Pre] [Pro] [F] [Catalytic Zn⁺⁺] [Hemopexin]	Laminin, fibronectin
Gelatinase A	MMP-2 EC 3.4.24.24	[Pre] [Pro] [Catalytic] [FN] [Zn⁺⁺] [Hemopexin]	Gelatins, collagens I, IV, V, VII, X, elastin, fibronectin
Gelatinase B	MMP-9 EC 3.4.24.35	[Pre] [Pro] [Catalytic] [FN] [Zn⁺⁺] [C] [Hemopexin]	Gelatins, collagens IV, V, elastin
MT1-MMP	MMP-14	[Pre] [Pro] [F] [Catalytic Zn⁺⁺] [Hemopexin] [TM]	Gelatins, fibronectin, laminin B, vitronectin, DSPC, Gelatinase A
MT2-MMP	MMP-15	[Pre] [Pro] [F] [Catalytic Zn⁺⁺] [Hemopexin] [TM]	Unknown
MT3-MMP	MMP-16	[Pre] [Pro] [F] [Catalytic Zn⁺⁺] [Hemopexin] [TM]	Gelatinase A
MT4-MMP	MMP-17	[Pre] [Pro] [F] [Catalytic Zn⁺⁺] [Hemopexin] [TM]	Unknown

Abbreviations: F - Furin consensus site; FN - Fibronectin-like domain; C - Collagen-like domain
TM - Transmembrane domain; Zn⁺⁺ - Zinc binding domain

Fig. 1. The members of the MMP family grouped according to domain structure. The accepted name of each MMP as recommended by the Enzyme Commission (EC) and used within the text, the MMP number, and the EC are given. The protein structure is outlined for each MMP with the five common domains as well as specific domains for a few distinct MMPs. The substrates listed are representative for each enzyme and are not necessarily comprehensive [see Chambers and Matrisian (27) for review].

surface. Four members of the MT-MMP family have been identified thus far, MT1-MMP, MT2-MMP, MT3-MMP, and MT4-MMP. In addition to the transmembrane domain, the MT-MMPs, along with stromelysin-3, contain a short region following their pro domains that corresponds to a consensus site for intracellular cleavage by furin-like enzymes.

The MMP family of enzymes can also be classified by their ability to degrade extracellular matrix components under physiological conditions (see Fig. 1). The collagenases are the only enzymes capable of degrading fibrillar collagens. Once the fibrillar collagens are cleaved into one-fourth and three-fourth fragments by the collagenases, other proteases such as

the gelatinases, A and B, may further degrade these substrates. The family members that can degrade a broad range of matrix proteins, glycoproteins, and proteoglycans are sometimes referred to as stromelysins. Subtle differences in substrate specificity within this subfamily have been observed. For example, metalloelastase and matrilysin, but not the stromelysins, can degrade elastin, and matrilysin has the most potent proteoglycanase activity of the MMPs tested. Stromelysin-3, on the other hand, is an exceptionally weak protease with limited substrates. MT1-MMP, one of the MT-MMP subfamily members, was initially identified as the cell-surface activator of gelatinase A. It has also been shown *in vitro* to proteolyze several types of matrix proteins readily including gelatin, fibronectin, the B chain of laminin, vitronectin, and dermatan sulfate proteoglycan (2), and most recently types I, II, and III collagen (3).

MMPs are usually secreted as soluble latent pro-enzymes, then activated in the extracellular environment by a mechanism termed the "cysteine switch" (4). A cysteine residue in the conserved PRGVDV sequence at the carboxy-terminal end of the pro domain, and three histidine residues in the HEXGHXXGXXH region of the catalytic domain, are essential for zinc binding; they maintain the inactive state of the enzymes. Disruption of the cysteine-zinc complex, usually by limited cleavage of the pro domain, results in a conformational change of the enzyme. Spontaneous opening of the "switch" leads to autolysis of the entire pro domain, yielding a proteolytically truncated and catalytically competent enzyme.

The activation of latent MMPs is affected by a variety of natural and synthetic molecules [(1) for review]. For example, plasmin activates most MMPs by cleaving once in the pro domain producing an unstable intermediate form of the enzyme which then autolyses to produce a fully active enzyme. Other enzymes such as cathepsin G, neutrophil elastase, trypsin, chymotrypsin, and plasma kallikrein, have also been shown to activate latent MMPs by similar mechanisms. The addition of organomercurials such as 4-aminophenyl-mercuric acetate (APMA), which have no intrinsic ability to cleave peptide bonds, destabilizes the MMP molecule and induces activation by disassociation of the zinc and the cysteine residue.

Once activated, the MMPs are susceptible to inhibition by tissue inhibitors of metalloproteinases (TIMPs). TIMPs are naturally occurring inhibitors of MMPs, they are expressed in most tissues and bind to

their active target in a 1:1 noncovalent bimolecular complex to inhibit their proteolytic activity [(1) for review]. Four members of the TIMP family have been identified thus far, TIMP-1, TIMP-2, TIMP-3, and TIMP-4 (5). In addition to binding to active MMPs, TIMPs may also bind to latent MMPs: TIMP-1 interacts with pro-gelatinase B, and TIMP-2 with pro-gelatinase A. TIMP-3 and TIMP-4 are not as well characterized but have been shown to possess MMP inhibitory activities. The balance between the levels of activated MMPs and TIMPs is thought to be at least one level of control that determines the integrity of extracellular matrix and basement membrane structures.

MMP EXPRESSION IN THE NORMAL MAMMARY GLAND

The mammary gland undergoes most of its morphogenesis in the juvenile and adult animal. Growth of the end bud and ductal elongation through adipose stroma require extensive remodeling of the extracellular matrix, as do the dramatic changes that accompany alveolar development during pregnancy and the return to a resting state upon the cessation of lactation. Consequently, this organ provides a unique opportunity for direct *in vivo* study and analysis of the role that MMPs may play in mediating morphological changes and regulating tissue function.

The expression pattern of MMPs in normal human breast tissue from reduction mammoplasties has been examined in a few reports. The expression of stromelysin-3 (6,7) and collagenase-3 (8) was negative in normal human breast tissue. Matrilysin mRNA and protein, on the other hand, were found in the epithelial cells of atrophic nonlactating human mammary gland (9), while gelatinase A was found in myoepithelial cells of normal and hyperplastic breast tissue (10). Due to the difficulty in obtaining normal human breast tissue, the dynamic changes that occur in the mammary gland are best studied in animal model systems. Therefore, this review will be focused primarily in studies on the murine mammary gland.

MMP Expression During Development, Pregnancy, Lactation, and Involution

The development of the murine mammary gland occurs in several distinct stages regulated by ovarian

hormones. Beginning about 3–4 weeks of age, with the onset of ovarian function, the terminal end buds at the ductal tips grow outward with an increasing rate and degree of branching [see (11) for review]. The end buds, highly mitotic and multilayered epithelial structures, serve as growth points for the elongation and branching of new ducts. By approximately 8–10 weeks of age, the entire fat pad is filled with an extensively branched network of epithelial ducts that are surrounded by a fibrous sheet of extracellular matrix, a basement membrane, a zone of loose connective tissue and an adipose fat pad. Temporal expression of several MMP family members correlates with the normal ductal branching morphogenesis that occurs during the development of the murine mammary gland. Between 5- and 10-weeks of age, when the mammary ducts are actively growing and branching, stromelysin-1 (12) and gelatinase A (12,13), are detected at very high levels; they decrease by 13 weeks of age in virgin adults. Stromelysin-3 and TIMP-1 mRNAs are also detected during this time period, but at much lower levels (12). Stromelysin-1 has been localized by *in situ* hybridization to vimentin-positive fibroblasts in the stroma surrounding the growing mammary duct (Fig. 2), but not to the region adjacent to the terminal end bud (12). This localization pattern of stromelysin-1



Fig. 2. Three-dimensional reconstruction of a branching mammary duct. Serial 5- μ m sections representing 100 μ m of an inguinal mammary gland were processed for *in situ* hybridization using an antisense mouse stromelysin-1 probe. Three-dimensional reconstruction of the mammary gland and stromelysin-1 localization was accomplished using Voxel View computer analysis. The ductal epithelium is depicted in blue and the *in situ* hybridization signal in red. Note that stromelysin-1 mRNA is particularly dense in fibroblasts located at the cleft point between a vertical large duct and a smaller branching duct [reprinted with permission Witty *et al.* (12)].

suggests a role in matrix repair after ductal growth has occurred. Gelatinase A and stromelysin-3 mRNA also localize to stromal cells, although the precise cell type has not been identified.

The major phase of mammary growth and differentiation occurs during pregnancy, culminating in lactation. This phase involves rapid and intense proliferation of the mammary epithelial cells followed by alveolar differentiation. During pregnancy the mammary epithelial cells increase by approximately 8–12 times their original number [see (11) for review]. Similarly to mammary development at puberty, several MMPs are detected in the mammary gland during early alveolar morphogenesis. Stromelysin-1 (12, 14), gelatinase A (12), and lower levels of stromelysin-3 (12) mRNA are expressed in the mammary gland during early pregnancy with peak levels of stromelysin-1 and gelatinase A occurring at day 6 of pregnancy. Stromelysin-1, as well as gelatinase A, is also localized by *in situ* hybridization to fibroblast-like cells in the stromal component surrounding the developing alveoli of the pregnant mammary gland (12).

After cessation of lactation the lobulo-alveolar structures of the mammary ducts undergo involution. The involution process is characterized by dramatic changes in the structure of the extracellular matrix, including degradation of the basement membrane [see (15) for review], as well as a loss of secretory epithelial cells which proceeds mainly by programmed cell death. The end result is a return to the resting, nonfunctional state of the mammary gland.

Several MMPs and their inhibitors have been implicated in the degradation and remodeling of the mammary stroma during involution. Gelatinase A (16, 17) and stromelysin-1 (16–18) are detected by approximately day four of involution and maintain their expression until day 10 (16), while interstitial collagenase is detected three days after weaning and persists at low levels for approximately 2 weeks (19). Investigators have suggested that the basement membrane surrounding the mammary epithelium is synthesized by stromal and/or myoepithelial cells (20, 21), and that the myoepithelial cells are responsible for its degradation as well (10, 17). In fact, gelatinase A (17, 22) and stromelysin-1 (17, 18, 22) protein have been reported to localize to the basal myoepithelial cells of the rodent mammary gland suggesting they may be responsible for degradation of the epithelial basement membrane during involution. TIMPs are also detected with peak expression at 4–6 days of involution (16, 18). When slow-release TIMP pellets are surgically implanted

into murine mammary glands, involution is delayed (16). These data emphasize that the time and the level of expression of MMPs and TIMPs must be tightly regulated, and suggest that the balance of extracellular matrix-degrading enzymes and their inhibitors control the rate of remodeling of the basement membrane that accompanies involution.

The epithelial-specific MMP, matrilysin, is also detected in the adult cycling, lactating, and involuting murine mammary gland (23). However, matrilysin mRNA levels are very low and can be detected by RT-PCR but not northern blotting or *in situ* hybridization (23). In presumably normal human breast tissue, however, abundant matrilysin mRNA and protein has been detected and localized to the mammary epithelial cells (9). The reason for the difference in expression levels is not clear, but it has been suggested that matrilysin may act as an "enzymatic pipe cleaner" to keep ducts patent (9). Physiological or environmental differences related to this activity may therefore account for the differences in the degree of matrilysin expression in murine and human tissues.

Roles for MMPs in Normal Cellular Processes: Proliferation, Differentiation, and Apoptosis

The expression patterns of MMPs suggest that they play an important role in the dramatic morphological and functional changes that take place in the mammary gland. However, it is not always clear whether MMP expression is the cause or the result of connective tissue remodeling. The targeting of MMPs to mammary tissue in transgenic mice has provided model systems to study the roles of these enzymes in tissue remodeling during mammary gland development and differentiation. Both the mouse mammary tumor virus (MMTV) and whey acidic protein (WAP) promoters have been used to target MMPs to the mammary epithelium. The MMTV promoter normally targets expression of the transgene to epithelial cells during ductal development and pregnancy (24) while the WAP promoter is expressed most abundantly during mid to late pregnancy and lactation (25).

Stromelysin-1 was been targeted to the mammary gland using the MMTV (12), and the WAP promoters (14). In both cases, a mutated form of the rat stromelysin-1 cDNA was used so that an active form of stromelysin-1 was produced constitutively. Overexpression of active stromelysin-1 increased the number of epithelial cells and the proliferative index in young, virgin

females (12, 14), (Table I). As a result, the developing mammary glands showed premature branching and an increase in alveolar structures (Fig. 3C–3F). At 8–10 weeks of development the mammary tree resembled a 9–12 day pregnant gland. In addition, expression of active stromelysin-1 resulted in the loss of laminin and type IV collagen (14) and disruption of the basement membrane (12), (Fig. 4) at times consistent with the expression of the tissue-specific promoter. It is unclear if the degradation of the basement membrane induces the alveolar phenotype directly, or if these changes are regulated by normal hormonal changes but the presence of the MMP prevents normal remodeling at the end of the cycle.

The relationship between MMPs and cellular proliferation is unclear. Although expression of stromelysin-1 in transgenic mice results in an increased number of epithelial cells and higher proliferative index, it is not known if this is a direct or an indirect effect. MMPs are induced by many growth factors and oncogenes that increase the proliferative index of cells and may thus participate in a growth response (26). Additional evidence for a role for MMPs in cellular growth comes from the use of MMP inhibitors on breast tumors [(27) for review]. Specifically, synthetic MMP inhibitors have been shown to reduce the growth of breast tumors *in vivo* (28, 29), although these inhibitors have little or no effect on the growth of tumor cells in monolayer culture (30). The mechanism of the growth stimulating effect of MMPs is not clear; it could be mediated through effects of matrix degradation products, the release of growth factors from matrix stores, or the cleavage and activation of resident growth factor [(26) for review]. More indirect effects on tumor angiogenesis have also been implicated in the case of the *in vivo* studies [(31) for review].

Bissell and colleagues have demonstrated that basement membrane components possess information critical to the differentiation of mammary epithelial cells (32, 33). In addition to an increase in mammary epithelial cell proliferation, the mammary glands of virgin stromelysin-1 transgenic animals expressed β -casein mRNA, normally consistently expressed only during pregnancy and lactation (12, 14), (Table I). MMP expression may therefore contribute to changes in the differentiation state of the mammary gland by its ability to alter basement membrane and composition.

The basement membrane is considered a survival factor for epithelial cells, and loss of contact between epithelial cells and basement membrane results in a specialized form of apoptosis referred to as anoikis

Table I. Comparison of Metalloproteinase Transgenic Animals

Transgenic	Lobuloalveolar development	Proliferation	Apoptosis	β -casein expression	Tumorigenesis
WAP-Stromelysin-1 Rat activated ^a	yes	Approx. Six-fold increase ^b (Number of alveolar-like buds)	10–15% increase ^c	mRNA	tumors
MMTV-Stromelysin-1 Rat activated ^a	yes	1.3-fold increase ^b (BrdU incorp.)	3.73-fold increase ^b	mRNA	No tumors; not analyzed for HANs
MMTV-Matrilysin Human wildtype	no	no difference	no difference	protein	HANs

^a Activated rat Stromelysin-1 contains mutations in either the Val(92) to Gly(92) or Pro(93) to Val(93).

^b Measured during mammary development.

^c Measured during mid-pregnancy.

(34, 35). When mammary epithelial cells are placed in culture without critical basement membrane or serum components, they undergo apoptosis which can be prevented by plating on exogenous or endogenous basement membrane proteins (36,37). In addition, disruption of cell-matrix interactions with antibodies against β_1 integrins results in apoptosis of cultured mammary epithelial cells (36). *In vivo*, the overexpression of an activated form of stromelysin-1 in the mammary gland of transgenic mice results in loss of basement membrane components (14), and a marked disruption of basement membrane structure (12), (Fig. 4). Consistent with a role for MMPs in modulating programmed cell death via alterations in the basement membrane, these mice show a significant increase in the apoptotic index in mammary tissue (36,38), (Table I). In addition, the alveoli of 8-day lactating stromelysin-1 transgenic mice appear smaller and convoluted with an occasional loss of central lumina (14), thus demonstrating characteristics of an involuting gland compared to the lactating glands of a wildtype mouse. These observations were further supported through the use of TIMP-1 transgenic mice. Introduction of the TIMP-1 transgene into WAP-stromelysin-1 mice rescued the mammary epithelial cells from apoptosis (39).

Not all MMPs have effects similar to those of stromelysin-1 on the proliferation, differentiation, and apoptosis of murine epithelial cells. In contrast to the results with the MMTV- and WAP-stromelysin-1 mice, overexpression of the wildtype or activated human matrilysin protein under the control of the MMTV promoter had very little or no effect on the general morphological development of the mammary ductal tree (40), (Fig. 3G and 3H). In addition, no significant

difference in the proliferative or apoptotic indices in virgin transgenic mice were observed (Table I). However, virgin MMTV-matrilysin transgenic mice displayed ectopic expression of a pregnancy-associated protein, β -casein, while the MMTV-stromelysin-1 (12) and WAP-stromelysin-1 (14) transgenic animals expressed β -casein mRNA but not protein and non-transgenic virgin mice had no detectable β -casein (Table I). Matrilysin is not required for normal mammary gland function or morphology, however, as there is no obvious effect of ablating the matrilysin gene on the mammary gland [(41) and unpublished observations]. The lack of the morphological features of lobuloalveolar development but the production of β -casein protein in virgin MMTV-matrilysin mammary glands implies that differentiated gene expression can be dissociated from the morphological changes that accompany functional differentiation of the mammary gland. β -casein expression, and not epithelial cell proliferation, may therefore be directly related to alterations in the integrity of the basement membrane of mammary epithelial cells.

There are several potential explanations for the differences between the stromelysin-1 and matrilysin transgenic mice. Experimental variation, such as differences in the integration sites, expression levels, and genetic backgrounds of the mice may be contributing factors, although the phenotypes were observed in several independent lines of mice in all cases. Stromelysin-1 contains a hemopexin/vitronectin-like domain absent in matrilysin, and may confer additional activities or alter substrate specificity *in vivo* resulting in the observed phenotypic differences. The abnormal tissue-type expression of stromelysin-1 in glandular epithelial

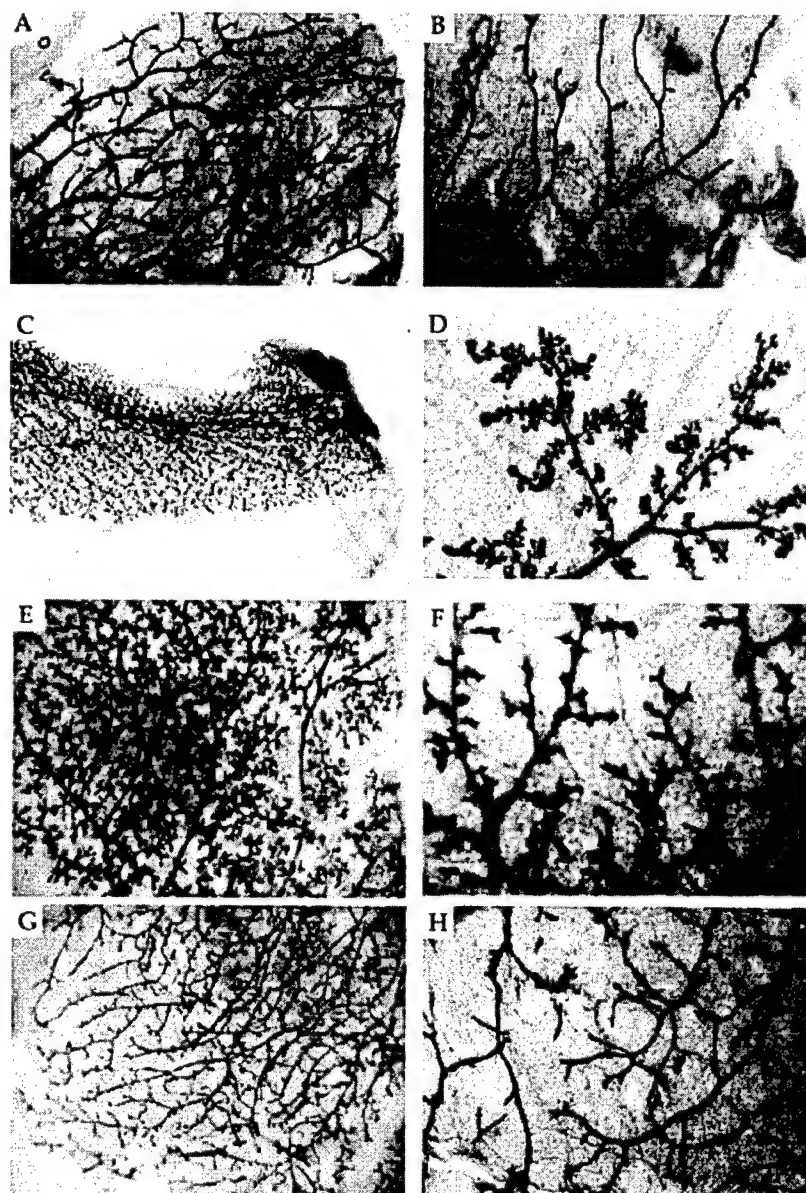


Fig. 3. Morphological appearance of normal and MMP transgenic virgin mammary glands. Iron hematoxylin-stained whole mounts of virgin mammary glands during various stages of development at low (A, C, E, G) and high (B, D, F, H) power magnifications. (A-B) Normal 12 week virgin; (C-D) WAP-stromelysin-1 10 week transgenic; (E-F) MMTV-stromelysin-1 12-13 week transgenic; (G-H) MMTV-matrilysin 12 week transgenic. (Panels E and F reprinted with permission, Witty *et al.* (12) Panels C and D provided by Z. Werb, UCSF).

cells, as opposed to its normal localization to stromal fibroblast cells surrounding the developing ducts (12) may also account for the more profound cellular alterations in these mice compared to matrilysin transgenic animals. In addition, the differential endogenous expression levels of stromelysin-1 and matrilysin in the mammary gland suggest that these MMPs may

have distinct roles during mammary development. The low endogenous expression levels of matrilysin (23) imply that this particular MMP plays a minor role in mammary development compared to the abundantly expressed stromelysin-1 (12). The less dramatic consequences of matrilysin overexpression lend weight to this conclusion.

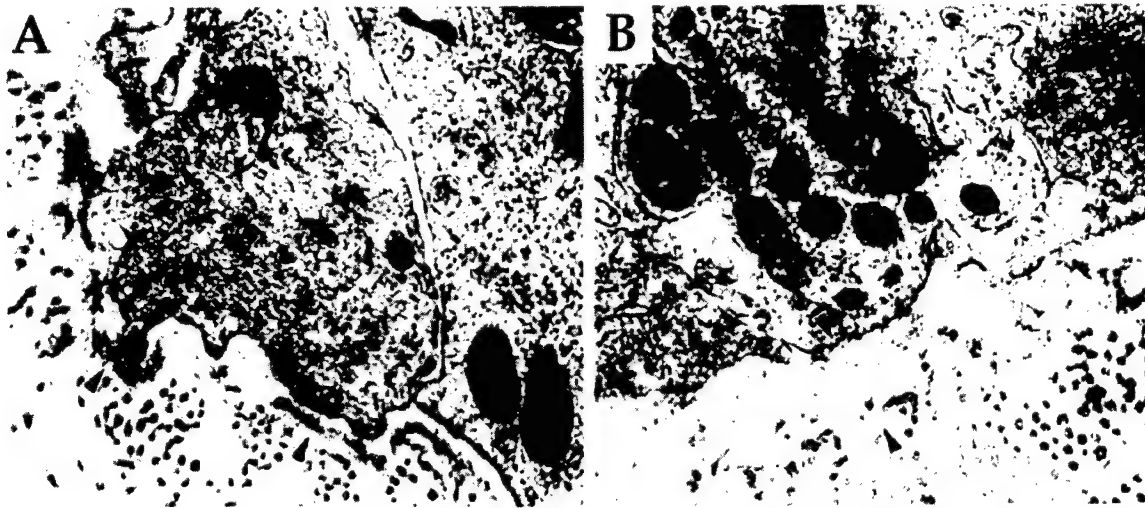


Fig. 4. Ultrastructure of normal and MMTV-stromelysin-1 mammary tissue. Transmission electron micrographs of mammary tissue from (A) normal; and (B) MMTV-stromelysin-1 transgenic 11 week virgin mammary glands. At this magnification the basal lamina resolves as two components: the lamina densa (at arrowheads) and lamina lucida (clear area adjacent to the epithelial cell border). In the transgenic tissue, the epithelial basement membrane appears less organized (area between two arrowheads) and has an amorphous region that lacks a distinct lamina lucida and densa [reprinted with permission, Witty *et al.* (12)].

MMPS IN MAMMARY TUMORS

One step required for tumor cells to metastasize involves the ability to invade the surrounding tissue, crossing the basement membrane to be transported to a secondary site. MMPs, because of their expression in late-stage tumors and their ability to degrade several different components of the extracellular matrix, are candidate enzymes to facilitate tumor cell invasion and metastasis. There is substantial experimental data to support a causal role for MMPs in these processes, and more recently, a role in early-stage tumor growth as well (27). The expression and role of MMPs in mammary carcinogenesis specifically is discussed later.

MMPs in Human Breast Cancer

MMPs expression in malignant breast disease is an expanding area of research. We will not attempt to cover this subject completely but will concentrate on the most significant recent contributions.

Stromelysin-3 has been shown to be expressed in a very high percentage of invasive breast cancers (6, 42–45), and has been detected within *in situ* carcinomas which are known to often become invasive tumors (6). In breast carcinomas, like many other types of human cancer, stromelysin-3 gene expression has been

shown to be restricted to the fibroblastic cells immediately surrounding the neoplastic cells (42); it was not found in the malignant cells themselves or in stromal cells at sites distant from the malignancy (44). The overexpression of stromelysin-3 correlated with poor outcome and was determined to be an independent prognostic marker (43).

Gelatinase A is also highly expressed in malignant mammary tumors, but has also been detected in normal tissue and benign lesions (10, 42, 46–49). Gelatinase A mRNA was detected in stromal fibroblast-like cells and differed from stromelysin-3 expression because it was found more widely throughout the tumor stroma [(50) for example]. Interestingly, however, gelatinase A protein showed a different pattern of expression. In normal breast tissue, gelatinase A protein was associated with the myoepithelial cells of lobules and ducts and was localized to tumor cells in either cytoplasmic or plasma membrane compartments in benign and malignant tumors (10, 46). These observations suggest that gelatinase A is produced primarily by stromal fibroblasts with the protein migrating and attaching to breast epithelial cell membranes. It is also possible that low levels of gelatinase A mRNA are made by breast epithelial cells, and that there are significant differences in protein localization and/or stability between epithelial cells and fibroblasts.

The actual significance of gelatinase A expression in breast cancer is currently disputed. In some studies,

the activity or expression of gelatinases did not correlate with metastatic progression or disease outcome (51). However, others have determined that the presence of activated gelatinase A correlates with disease progression (52). Gelatinase A activation appeared to involve a complex of a membrane type MMP, TIMP-2, and progelatinase A (53–55). Interestingly, the levels of TIMP-2, an MMP inhibitor, which binds to the COOH-terminus of progelatinase A, correlated with the clinical outcome of breast disease (56). In light of these observations, it was initially surprising that MT1-MMP mRNA was reported to localize to stromal cells in breast carcinomas (50, 57, 58) rather than to tumor epithelium as anticipated. However, gelatinase A activation correlated with an epithelial-to-mesenchymal transition by breast cancer cell lines in culture, and constitutive expression of MT1-MMP was observed in cell lines that had undergone this transition (59). MT2-MMP and MT4-MMP have also been detected in some breast tumors and tumor cell lines, but the existing evidence points to a role for MT-MMPs in gelatinase A activation in breast cancer (57, 60, 61).

Human breast lesions have also been examined for matrilysin expression. Matrilysin mRNA expression was detected in 70–90% of invasive ductal carcinomas as determined by northern analysis (6, 42) and localized by *in situ* hybridization to neoplastic epithelial tumor cells (50). In addition, matrilysin mRNA was found in normal and preneoplastic mammary epithelium. 100% of benign fibroadenomas and ductal carcinoma *in situ* specimens examined also expressed matrilysin mRNA (6, 42). As previously outlined, matrilysin can be detected at low levels in the normal human breast tissue surrounding neoplastic tissue (6), and has been observed in the normal tissue from breast reductions (9). Matrilysin protein is localized apically and lumenally in normal mammary epithelium (9), suggesting that the activity of this MMP extends much farther than degradation of matrix components during tumor cell invasion and is likely to include maintenance of normal ductal functions.

Roles for MMPs in Mammary Tumorigenesis

The expression of MMPs and their inhibitors in murine models of mammary tumorigenesis have been examined in a limited number of studies. Stromelysin-1 protein and TIMP-1 mRNA have been found in the myoepithelial cells surrounding pre-neoplastic lesions in the mammary tumors produced by WAP-*ras*

transgenic animals, but were absent in the nonmetastatic tumors produced by the WAP-*c-myc* transgenic animals (18). At advanced stages of the WAP-*ras* induced metastatic tumors, stromelysin-1 was also expressed in the tumor cells as well as the surrounding stroma, perhaps reflecting an epithelial-to-mesenchymal transition as occurs in late stage breast cancers. Mammary tumors induced by 7,12-dimethylbenzanthracene (DMBA) treatments in MMTV-stromelysin-1 and MMTV-TGF α transgenic animals also express endogenous mouse stromelysin-1 in the stromal tissue surrounding noninvasive or focally invasive adenocarcinomas (38). These studies establish that murine mammary tumors express MMPs and their inhibitors and provide a model system for examination of the roles of specific MMPs on various stages of mammary tumor progression.

MMPs and their inhibitors have been altered genetically to examine a role for MMPs in mammary tumorigenesis. The overexpression of TIMP-4 in human MDA-MB-435 breast carcinoma cells reduced both *in vitro* invasiveness using the Boyden chamber assay and *in vivo* metastasis in nude mice (62). The overexpression of TIMP-4 had a negative influence on the growth of MDA-MB-435 tumors as well as on angiogenesis when assayed by histological microvessel counts. Expressing mouse or human stromelysin-3 in human breast-derived MCF-7 cells increased the efficiency and time of establishment of tumors in nude mice, while decreasing endogenous levels of stromelysin-3 in mouse fibroblasts using antisense technology reduced their tumorigenicity (63). However, there was no difference in the metastatic ability of tumors in these model systems, pointing to a role for stromelysin-3 in early stage tumor development rather than in the late stages of tumor invasion and metastasis. Mammary tumor-derived cells expressing an activated form of rat stromelysin-1 displayed an enhanced invasive ability *in vitro* (64). The invasive phenotype could be inhibited through the use of a specific MMP inhibitor but not inhibitors of other protease classes. Antisense oligonucleotides against stromelysin-1 but not collagenase-3 or stromelysin-3 were also found to mimic the effect of the MMP inhibitor in this model system. In these model systems the stromal MMP was overexpressed in the epithelial cell compartment. Presumably, however, the effect of the MMP on malignant epithelium would be the same irrespective of the source of the protein.

Mammary tumor studies in stromelysin-1 transgenic animals have produced conflicting results

(Table I). WAP-stromelysin-1 animals were reported to develop spontaneous phenotypic abnormalities ranging from hyperplasia to adenocarcinoma (65), providing dramatic evidence for a role of MMPs in mammary tumorigenicity. Studies utilizing MMTV-stromelysin-1 animals, on the other hand, showed a reduction in the number of mice developing mammary tumors following treatment with the chemical carcinogen DMBA (38). This effect was associated with a three to fourfold increase in the number of apoptotic cells in the MMTV-stromelysin-1 transgenic mammary gland at the time of DMBA administration (38). These results are likely to represent an effect of stromelysin-1 on the target of DMBA action rather than an effect on cells predisposed to malignancy. The conflicting results may therefore be a consequence of differences in the mechanism of tumor induction in these studies.

Recently, MMTV-matrilysin transgenic animals were evaluated for a potential role for matrilysin in mammary tumorigenesis by mating these animals to MMTV-*neu* transgenic mice. There was a small but not statistically significant increase in the percentage of mice with lung metastasis in the MMTV matrilysin/*neu* mice. However, the MMTV-matrilysin/*neu* mice showed a dramatic acceleration in the onset of mammary tumors (66). In addition, approximately 50% of multiparous MMTV-matrilysin transgenic animals developed preneoplastic mammary lesions. These distinctive focal areas of epithelial hyperplasia have been previously termed hyperplastic alveolar nodules (HANs), and are considered to be premalignant precursors prone to develop into mammary carcinomas (67,68). Based on these results and the expression of matrilysin in human breast tissue (9,50), a role in very early stages of tumor progression can be postulated for this MMP.

Synthetic MMP inhibitors have also been tested in several breast cancer models. The broad-spectrum MMP inhibitor batimastat reduced the development of metastatic lesions in models for either hematogenous or lymphatic spread of breast cancer cells (29). In an experimental model of breast cancer bone metastasis, TIMP-2 alone or in combination with an inhibitor of bone resorption resulted in a decrease in detectable bone lesions (69). In a few cases, the inhibitor effected the ability of the metastatic lesions to grow at ectopic sites (29). Intravital microscopy studies in which TIMP-1 was demonstrated to alter the growth of injected tumor cells rather than the ability of the cells to extravasate are consistent with this observation (70). In addition to effects on metastatic lesions, batimastat

also reduced the growth of primary tumors following surgical resection (28). MMP inhibitors have also been demonstrated to have anti-angiogenic effects which may indirectly effect the growth of both primary tumors and metastatic lesions [(31) for review]. These results demonstrate that MMPs may contribute to the malignant phenotype in both early and late stages of tumor progression.

CONCLUSIONS

The expression pattern of MMPs in the normal mammary gland and in human and murine models of breast cancer suggest a role for these matrix-degrading enzymes in the dynamic changes that occurs in this tissue. Experimental evidence for these roles is gradually accumulating. In particular, the development of transgenic mouse models in which MMPs and TIMPs are targeted to the mammary gland has provided evidence for a role for MMPs in alterations in cellular proliferation, differentiation, and apoptosis. In addition, a contribution of specific MMPs to both early and late stages of mammary tumor progression has been documented. The stage has been set for further studies using MMP-deficient mice as well as specific synthetic MMP inhibitors to provide even more detail on the cellular and molecular effects of individual MMPs. The mammary gland provides an ideal system in which to dissect the effects of MMPs in a functionally versatile biological system with relevance to both normal and pathological processes.

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Overexpression of the Matrix Metalloproteinase Matrilysin Results in Premature Mammary Gland Differentiation and Male Infertility

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To examine the role of matrilysin (MAT), an epithelial cell-specific matrix metalloproteinase, in the normal development and function of reproductive tissues, we generated transgenic animals that overexpress MAT in several reproductive organs. Three distinct forms of human MAT (wild-type, active, and inactive) were placed under the control of the murine mammary tumor virus promoter/enhancer. Although wild-type, active, and inactive forms of the human MAT protein could be produced in an in vitro culture system, mutations of the MAT cDNA significantly decreased the efficiency with which the MAT protein was produced in vivo. Therefore, animals carrying the wild-type MAT transgene that expressed high levels of human MAT in vivo were further examined. Mammary glands from female transgenic animals were morphologically normal throughout mammary development, but displayed an increased ability to produce β -casein protein in virgin animals. In addition, beginning at approximately 8 mo of age, the testes of male transgenic animals became disorganized with apparent disintegration of interstitial tissue that normally surrounds the seminiferous tubules. The disruption of testis morphology was concurrent with the onset of infertility. These results suggest that overexpression of the matrix-degrading enzyme MAT alters the integrity of the extracellular matrix and thereby induces cellular differentiation and cellular destruction in a tissue-specific manner.

INTRODUCTION

The matrix metalloproteinases (MMPs)¹ are a family of extracellular proteases thought to be responsible for normal matrix remodeling and pathological tissue destruction by virtue of their ability to catabolize extracellular matrix components (Birkedal-Hansen *et al.*, 1993; Hulboy *et al.*, 1997 for review). The expression pattern of MMPs in normal tissues suggests that they are particularly involved in the remodeling associated with reproductive processes, including menstruation, trophoblast invasion, mammary gland morphogene-

sis, and involution of the uterus, mammary gland, and prostate (Hulboy *et al.*, 1997). Studies with natural and synthetic inhibitors of MMPs (Brannstrom *et al.*, 1988; Butler *et al.*, 1991; Talhouk *et al.*, 1992; Marbaix *et al.*, 1996) and recent experiments that utilized genetically altered mice that express altered MMP substrates (Liu *et al.*, 1995) or lack specific MMP family members (Rudolph-Owen *et al.*, 1997) have confirmed the importance of MMPs in reproductive processes in several systems.

Sixteen MMP family members have been described (reviewed in Hulboy *et al.*, 1997; see also Cossins *et al.*, 1996; Puente *et al.*, 1996). All MMPs described have three essential domains: a signal sequence or prodomain to direct secretion from the cell, a pro sequence to maintain latency, and a catalytic domain containing the critical zinc-binding site. Matrilysin (MAT, MMP-7, pump-1, uterine metalloproteinase, EC

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¹ Abbreviations used: MAT, matrilysin; MMP, matrix metalloproteinase; MMTV-LTR, murine mammary tumor virus long terminal repeat.

3.4.24.23) is unique in that it contains only these minimal domains. All other MMP family members have an additional hemopexin/vitronectin-like domain that is connected to the catalytic domain by a variable hinge region (Birkedael-Hansen *et al.*, 1993 for review). This and other domains found in specific MMP family members generate diversity by modifying properties such as substrate specificity, interaction with endogenous inhibitors of metalloproteinases, intracellular activation, and cell-surface localization (Powell and Matrisian, 1996 for review).

Matrilysin (MAT) is considered a member of the stromelysin subfamily of MMPs. The stromelysins, including stromelysin-1 (STR-1, MMP-3, EC 3.4.24.17), stromelysin-2 (STR-2, MMP-10, EC 3.4.24.22), and MAT, can degrade a broad range of substrates such as fibronectin, proteoglycans, and denatured and basement membrane collagens. Subtle differences in substrate specificity within this subfamily have been observed. For example, although extracellular matrix proteoglycans are substrates for MAT, STR-1, and STR-2, MAT can degrade elastin (Murphy *et al.*, 1991; Imai *et al.*, 1995), entactin (Sires *et al.*, 1993), and tenascin (Imai *et al.*, 1994; Siri *et al.*, 1995) more efficiently than the other stromelysins. MAT is also distinct from most MMP family members in that it is expressed primarily by normal and malignant glandular epithelial cells. MAT is expressed in the epithelial cells of the cycling human endometrium, small intestinal crypts, and postpartum and cycling mouse uterus, as well as epithelial tumors of the gastrointestinal tract, prostate, and breast (Wilson and Matrisian, 1996 for review). The stromelysins and most other MMP family members, in contrast, are expressed primarily in mesenchymal tissues, including endometrial stromal (Hulboy *et al.*, 1997 for review) and stromal cells surrounding several tumor types (Powell and Matrisian, 1996 for review). The unique protein structure, altered substrate specificity, and uncommon localization patterns of MAT suggest that this MMP may have *in vivo* functions that are distinct from other stromelysins and MMP family members.

Since MMPs are expressed in a variety of reproductive organs and there are features that distinguish MAT from other stromelysins and MMP family members, we were interested in determining the effects of MAT overexpression in reproductive organs. Transgenic mice expressing wild-type, inactive, and constitutively active MAT under the control of the murine mammary tumor virus (MMTV)-long terminal repeat (LTR) were generated, and their effect on mammary gland development and male reproductive function was assessed. The comparison of these transgenic mice with other MMP-expressing mice provides insights into the action of specific MMPs in reproductive processes.

MATERIALS AND METHODS

Plasmid Construction

The 1.1-kilobase (kb) full-length human MAT cDNA (pPump-1; Muller *et al.*, 1988) was altered by oligonucleotide-directed mutagenesis. An active form of human MAT with a substitution of valine to glycine at amino acid 92 was generated with the oligonucleotide 5'-CCGGTGTGGTGGGCCCGACGTC-3' and cloned into pKCR3 (Witty *et al.*, 1994). An inactive form of human MAT with a substitution for glutamic acid to glutamine at amino acid 216 was generated with the oligonucleotide 5'-ATGGCCAAGTTGATGAG-TTGC-3' and also cloned into pKCR3. The resulting full-length human MAT cDNAs (wild-type, active, and inactive, Figure 1B) were subcloned into the unique *EcoRI* site of the MMTV-LTR expression vector pMMTV-EV (Matsui *et al.*, 1990) to generate pMMTV-MAT, pMMTV-ActMAT, and pMMTV-InMAT, respectively (Figure 1A). The expression vector contains intron, splice sites, and polyadenylation signals derived from the rabbit β -globin gene that increase the efficiency of expression (Breathnach and Harris, 1983).

Human MAT cDNA probes were generated by digesting full-length human MAT in pPump-1 (Muller *et al.*, 1988) with *EcoRI* and *XbaI* to remove the poly(A)⁺ tail. The 1.1-kb human MAT fragment was then subcloned into pGEM7zf(+) to yield pG7 pumpEX. For *in situ* hybridization, a 356-base pair (bp) fragment of human MAT cDNA corresponding to positions +700 to +1056 was amplified by polymerase chain reaction (PCR) using the primers 5'-CGCGTCT-AGACCTCTGATCCTAATGCAG-3' and 5'-CGCGAAGCTTGAC-ATCTACGCGCACTG-3'. The human MAT fragment was subcloned into pGEM7zf(+) to yield pG7-HmatUT and linearized with *HindIII* or *XbaI* to generate riboprobe templates for transcription using either T7 (antisense) or SP6 (sense) RNA polymerase, respectively.

Generation of MMTV-MAT Transgenic Mice

The three human MAT constructs, pMMTV-MAT, pMMTV-ActMAT, and pMMTV-InMAT, were purified by CsCl centrifugation, and the *AatII*/*BglIII* fragment was then isolated by gel electrophoresis on low melting point agarose (SeaKem Agarose; FMC BioProducts, Rockland, ME) and purified using Gelase (Epicentre Technologies, Madison, WI). The purified fragments were subsequently injected into FVB/N fertilized eggs by the Vanderbilt Transgenic/ES Cell Shared Resource and transferred to pseudopregnant mothers as described (Hogan *et al.*, 1995). Transgenic founders were identified by Southern blot analysis of *EcoRI*-digested genomic tail DNA using a random-primed (DNA Labeling Kit; Boehringer Mannheim, Indianapolis, IN) 1.1-kb *EcoRI*/*XbaI* fragment of human MAT from pG7 pumpEX. The approximate copy number of founder transgenic animals was determined by adding 10 pg (1 copy) or 100 pg (10 copies) of MMTV-MAT to genomic DNA from a nontransgenic mouse and comparing relative intensity of hybridization. Transgenic lines were generated by mating founder animals with FVB/N nontransgenic mice.

Immunoprecipitation

The human breast cancer cell line Hs578t was transiently transfected with 10 μ g of pMMTV-MAT, pMMTV-ActMAT, or pMMTV-InMAT. The cells were allowed to recover overnight, placed in serum- and methionine-free media for 6 h, and labeled with 100 μ Ci of [³⁵S]methionine for 14–16 h. The MMTV promoter was induced with 100 μ M dexamethasone (Dex) in the culture media for 14–16 h before collection of the conditioned medium. The wild-type, mutant, and inactive MAT protein from 9×10^5 trichloroacetic acid precipitable counts was immunoprecipitated from ³⁵S-labeled conditioned medium using a polyclonal antibody raised against human MAT (McDonnell *et al.*, 1991) and separated on a SDS-polyacrylamide gel. In addition, the MAT protein in the conditioned medium was activated by incubation with 1 mM of the organic mercuride

4-aminophenyl-mercuric acetate (APMA) at 37°C for 30 min before electrophoresis.

Tissue Preparation

The right thoracic and inguinal mammary glands and other organs were removed and frozen in liquid nitrogen or on dry ice and stored at -70°C. Tissue was later homogenized in a guanidinium/acid phenol solution, and total RNA was extracted as described by Chomczynski and Sacchi (1987). Poly(A)⁺ RNA was then isolated from total RNA over an oligo dT cellulose (Collaborative Biomedical Products, Bedford, MA) column or a latex bead-oligo dT column (Oligotex; Qiagen, Chatsworth, CA).

Left thoracic mammary glands were routinely fixed overnight in 4% paraformaldehyde and PBS for whole mount staining, while the left inguinal mammary glands and the testis and epididymis were fixed in paraformaldehyde and embedded in paraffin for subsequent sectioning.

Northern Analysis

Three to four micrograms of poly(A)⁺ RNA were electrophoretically separated on a 1% agarose-formaldehyde gel, transferred to nitrocellulose membrane (Micron Separations, Inc., Westborough, MA), and UV cross-linked (Stratagene, La Jolla, CA). Blots were hybridized at 42°C under high-stringency conditions [50% formamide, 5× SSC, 1× PAF (50× = 10g each polyvinyl pyrrolidone, bovine serum albumin, and ficoll/1), 20 mM NaPO₄, 0.1% SDS, 50 μg/ml salmon sperm, and 4% dextran sulfate] using the radiolabeled, random-primed (DNA Labeling Kit; Boehringer Mannheim) 1.1-kb *EcoRI/XbaI* fragment of the human MAT cDNA, the 700-bp *ApaI/HindIII* fragment of the mouse MAT cDNA from plasmid pG7-mMATAH (Wilson *et al.*, 1995), or the cDNA for the endogenously expressed cyclophilin gene (1B15; Danielson *et al.*, 1988) to control for RNA loading. Washes were carried out at 50°C in 0.1× SSC and 0.1% SDS.

In Situ Hybridization

Paraffin-embedded paraformaldehyde-fixed tissue sections 5–7 μm in thickness were placed onto Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA) and analyzed for the human MAT transgene expression as previously described (McDonnell *et al.*, 1991). The slides were prehybridized for 2–4 h, after which ³⁵S-labeled riboprobes at 1.2 × 10⁶ cpm/slide were added and hybridized overnight at 50°C. The slides were dipped in photographic emulsion (type NTB2; Kodak, Rochester, NY), exposed for 2 to 4 wk at 4°C, developed, and counterstained with hematoxylin. Background hybridization was assessed using the sense probe for each transcript analyzed.

Whole Mount Analysis

Inguinal and/or thoracic mammary glands were removed and placed flat in plastic embedding cassettes (Fisher Scientific, Pittsburgh, PA), fixed in 4% paraformaldehyde in PBS overnight, transferred to 70% ethanol, and stored at 4°C. The glands were defatted in 100% acetone and stained with iron hematoxylin (0.1% wt/vol hematoxylin, 0.1 M FeCl₃, 0.17 M HCl in 95% EtOH) for 3 h (Medina, 1973). Whole mounted glands were destained in 0.025 M HCl in 50% ethanol, dehydrated to xylene, and stored in 100% methyl salicylate. Glands were viewed using a Nikon dissecting microscope (Southern Micro Instruments, Atlanta, GA).

Immunohistochemistry

Paraformaldehyde-fixed, paraffin-embedded sections were dewaxed, hydrated through graded ethanols, treated with 0.6% hydrogen peroxide in methanol (to destroy endogenous peroxidase activity), microwaved in 0.1 M sodium citrate for 3 min and 45 s at high power to unmask the antigens and exposed to blocking solu-

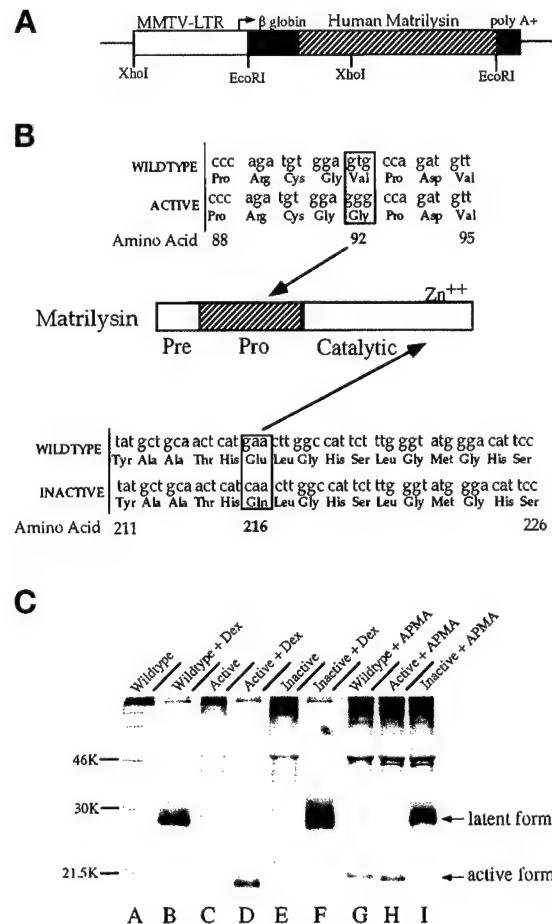


Figure 1. (A) MMTV-MAT transgenic construct. Diagram of the plasmid construct utilized for creation of transgenics. The filled boxes correspond to the 3'-splice sites and 5'-polyadenylation signals of the rabbit β-globin gene. Three forms of the human MAT cDNA were inserted into the third exon of the β-globin gene. (B) Mutations in the human MAT transgenic constructs. The MAT cDNA sequence and corresponding amino acids are depicted. Boxed areas indicate the position of the nucleotide mutation and amino acid substitution. Active MAT contains a substitution in the pro domain at amino acid 92 from valine to glycine, while inactive MAT contains a substitution in the catalytic domain at amino acid 216 from glutamic acid to glutamine. (C) Immunoprecipitation of the MAT protein. The breast cancer cell line Hs578t was transiently transfected with each MMTV-MAT construct, and the MAT protein immunoprecipitated from the conditioned medium as indicated by the arrows. Dex (100 μM) was added to the culture medium of samples shown in lanes B, D, and F. APMA, a known MMP activator, was added to the conditioned medium of samples shown in lanes G, H, and I.

tion (10 mM Tris, pH 7.4, 100 mM MgCl₂, 0.5% Tween-20, 1% wt/vol bovine serum albumin, and 5% wt/vol goat serum) for 1 h. Sections were incubated overnight at 4°C in blocking solution with affinity-purified rabbit anti-human MAT antibody (1:1000 dilution; kindly provided by Dr. William Parks, Washington University School of Medicine, St. Louis, MO; Saarialho-Kere *et al.*, 1995), or control rabbit IgG (Sigma Chemicals, St. Louis, MO). The sections were washed in TBST buffer (150 mM NaCl, 10 mM Tris, pH 8.0, and 0.05% Tween-20) and incubated with biotinylated anti-goat IgG

(1:5000; Vector Laboratories, Burlingame, CA) for at least 1 h at room temperature. Labeled cells were visualized using an avidin-biotin peroxidase complex (Vectastain ABC kit, Vector Laboratories) and TrueBlue peroxidase substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD). Sections were then counterstained with Contrast Red.

Tissues were similarly processed but without microwave treatment with a rabbit antibody to mouse casein (1:5000 dilution; kindly provided by Dr. Charles Daniel, University of California at Santa Cruz; Robinson *et al.*, 1993), or to proliferating cell nuclear antigen (1:100 dilution; Sigma; Waseem and Lane, 1990).

To localize the transgene protein in the testis and epididymis, tissue was dissected and frozen in OCT medium (Fisher Scientific) and liquid nitrogen. Five-micrometer sections were postfixed in Bouin's fixative and then washed in PBS for 10 min. Slides were dipped in saturated LiCO_3 to eliminate picric acid and washed again in PBS. Sections were analyzed for the expression of human MAT protein as described above for paraformaldehyde-fixed tissues except without microwave treatment.

Analysis of Programmed Cell Death

Paraffin-embedded sections were analyzed for apoptotic cells using a modification of the TUNEL assay (Gavrieli *et al.*, 1992). Tissues were deparaffinized, and endogenous peroxidases were quenched with 1% hydrogen peroxide in ethanol and incubated in chloroform to remove lipids and reduce background levels of staining, as previously described (Witty *et al.*, 1995a). Tissues were then dehydrated and washed with $1 \times$ TBS, and free 3'-OH DNA ends were labeled with biotin-conjugated deoxyribonucleotide triphosphate (Boehringer Mannheim) using terminal deoxynucleotidyl transferase (TdT) (Life Technologies BRL, Grand Island, NY), followed by a 1:5000 dilution of a horseradish peroxidase-streptavidin-conjugated antibody (Jackson Immuno Research Laboratories, West Grove, PA). Labeled cells were visualized with 1,2-diaminobenzidine and hydrogen peroxide, counterstained with hematoxylin, dehydrated through ethanol to xylene, and coverslipped under Permount.

RESULTS

Generation and Evaluation of MAT Expression Constructs

To determine the effects of ectopic expression of the metalloproteinase MAT on select reproductive organs, the human MAT cDNA was placed under the control of the MMTV-LTR in a vector designed to contain flanking, splice, and polyadenylation sites to improve expression efficiency (Figure 1A). This expression vector has been demonstrated to be effective in directing transgene expression to the murine mammary gland, salivary gland, brain, testes, and epididymis (Matsui *et al.*, 1990; Witty *et al.*, 1995b). Three distinct expression constructs were generated to produce wild-type, constitutively active, and inactive forms of the MAT protein (Figure 1B). The constitutively active MAT cDNA contains a valine-to-glycine substitution in the highly conserved sequence PRCGVPDV, which corresponds to amino acids 88–95 near the carboxyl-terminal end of the prodomain. Mutations in the rat stromelysin-1 sequence in this same region leads to variants that have a significantly increased tendency to spontaneously generate active STR-1 (Sanchez-Lopez *et al.*,

1988; Park *et al.*, 1991). MAT protein produced by this construct therefore theoretically circumvents any dependence on exogenous factors for activation. The catalytically inactive MAT cDNA contains a glutamic acid-to-glutamine substitution at position 216 within the highly conserved zinc-binding domain. Similar mutations in the rat STR-1 cDNA result in protein that cannot be activated by organic mercurides to auto-proteolyze (Sanchez-Lopez *et al.*, 1988). We reasoned that this construct would encode a MAT protein with three-dimensional structure that deviates only slightly from wild-type MAT but lacks proteolytic activity. This inactive mutant would provide a control to determine whether observable effects could be attributed to the catalytic activity of this metalloproteinase.

To determine whether the wild-type, active, and inactive human MAT transgenic constructs were functional *in vitro*, the human breast cancer cell line Hs578t was transfected with each MMTV-MAT expression vector, and the conditioned medium was analyzed for MAT protein by immunoprecipitation. Specific MAT immunoreactivity was not detected in the conditioned medium of untreated transfected cells, but was induced by the addition of the synthetic glucocorticoid Dex, a known inducer of the MMTV promoter/enhancer (Ringold, 1983; Figure 1C, compare lanes A and B, C and D, and E and F). The wild-type MAT cDNA produces a 28-kDa protein corresponding to the latent zymogen (Figure 1C, lane B). In the presence of the organic mercuride APMA, which activates the cysteine switch of MMPs (Birke-dael-Hansen *et al.*, 1993 for review), wild-type MAT protein was cleaved and converted to 19 kDa, consistent with the removal of the pro domain and conversion to the mature, active catalytic form (Figure 1C, compare lanes B to G). Immunoprecipitation of the constitutively active human MAT protein demonstrated that the majority of the protein in the medium of transfected cells was present in the 19-kDa active form, and the residual 28-kDa protein could be completely converted to the activated form with APMA treatment (Figure 1C, lanes D and H). These data indicate that the mutation in the pro domain of MAT results in constitutive activation of the enzyme in the absence of exogenous activators, as was predicted from the results of similar mutations in rat STR-1 (Sanchez-Lopez *et al.*, 1988; Park *et al.*, 1991). In contrast, the inactive mutant protein was produced as the higher molecular weight proenzyme form and was not converted to the mature form by APMA treatment, indicating that a mutation at amino acid 216 in human MAT prevents the enzyme from autoproteolytic cleavage and thus represents an inactivating mutation (Figure 1C, compare lanes F and I).

Generation of MAT-expressing Transgenic Mice

To test the effects of overexpressing the epithelium-specific matrix-degrading enzyme MAT in select reproductive tissues *in vivo*, the MMTV-MAT expression vectors producing wild-type, active, and inactive MAT protein were used to establish transgenic mouse lines by standard pronuclear injection techniques. Transgenic mice were identified by Southern blot analysis, and founder mice harboring the transgene were mated to establish transgenic lines (Figure 2). At least two lines per construct were established with varying copy numbers to control for insertional variation. The resulting transgenic lines were identified by the founder animal number and will be referred to hereafter as MMTV-MAT (MMTV-wild-type-matrilysin), MMTV-ActMAT (MMTV-active-matrilysin), and MMTV-InMAT (MMTV-inactive-matrilysin).

Expression and Localization of the MAT Transgene in Mammary Epithelial Cells

The MMTV-LTR promoter/enhancer has been used extensively to drive the expression of transgenes in the mammary epithelium (Cardiff and Muller, 1993 for review). The MMTV-LTR promoter activity responds to endogenous steroid hormone levels in the murine mammary glands during development, pregnancy, and lactation, as well as during the normal estrous cycle (Gunzburg and Salmons, 1992). MAT expression in the transgenic animals was analyzed by Northern blot analysis of poly(A⁺) RNA from developing mammary glands of female mice harboring the wild-type, active, and inactive human MAT constructs. We observed considerable variability in the expression of the MAT transgene within and between the various transgenic lines, presumably due to hormonal fluctuations. For example, approximately 42% (8/19) of transgenic mammary glands examined from MMTV-MAT line 3 expressed human MAT wild-type mRNA at various stages of mammary development. No correlation of human MAT mRNA expression could be made for a particular time during mammary development or during a specific day of the estrous cycle. However, when animals in which the MAT transgene was expressed were compared, human MAT expression appeared abundant between 6 and 17 wk of age in the MMTV-MAT lines 3 and 42 and was absent in nontransgenic littermate controls (Figure 3A and our unpublished results). MMTV-ActMAT lines 1 and 22 also displayed detectable levels of MAT mRNA during mammary gland development, although in general MAT mRNA levels appeared lower than for MMTV-MAT mice (Figure 3B). In contrast, MAT mRNA appeared as a smear instead of a distinct band when isolated from the mammary glands of both the MMTV-InMAT lines 2 and 4 animals (Figure 3C). Several attempts were made to extract intact human

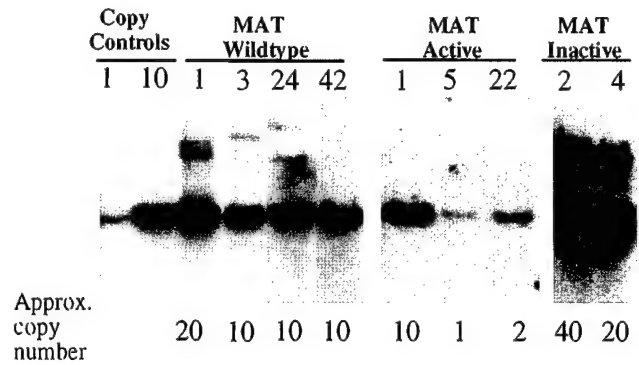


Figure 2. MMTV-MAT transgenic lines. Southern hybridization of 10 μ g of genomic DNA from MMTV-MAT founder animals (lines 1, 3, 24, and 42), MMTV-ActMAT founder animals (lines 1, 5, and 22), and MMTV-InMAT founder animals (lines 2 and 4). The approximate copy number of each line is indicated below the lane.

MAT RNA from the mammary glands of the MMTV-InMAT transgenic lines, all of which proved to be futile. Endogenous mouse MAT mRNA was not detectable by Northern blot analysis in the developing mammary glands of either nontransgenic or MMTV-MAT transgenic mice (our unpublished results). However, we have previously shown by reverse transcriptase-PCR that low levels of mouse MAT are expressed in the adult mammary gland (Wilson *et al.*, 1995).

Immunohistochemistry was performed to localize the product of the MAT transgene in the mammary glands. Protein expression from the MMTV-MAT wild-type transgene was detected during several stages of mammary gland development (6–14 wk), with staining localized to the cytoplasm of the epithelial cells of the mammary ducts (Figure 4A and data not shown). MAT protein was also detected in mammary tissue from the MMTV-ActMAT lines, but at relatively lower levels than the MMTV-MAT wild-type lines (Figure 4B). We detected no MAT immunoreactivity in mammary glands from the MMTV-InMAT animals (Figure 4C), suggesting that the mutation impairs the production of MAT protein *in vivo*. No immunoreactivity was detected in any mammary gland sections from nontransgenic littermate controls at various times of mammary development (Figure 4D for example). Because of the absence or low expression of MAT protein in the transgenic animals carrying the mutated human MAT cDNA constructs and the high MAT expression in the transgenic animals carrying the wild-type human MAT cDNA construct, we focused our attention in subsequent studies on those animals carrying the wild-type MAT transgene. In general, initial observations suggest that transgenic animals carrying the activated form of MAT showed similar phenotypes to wild-type MAT transgenics but to a lesser degree, while the inactive MAT transgenics were indistinguishable from nontransgenic controls.

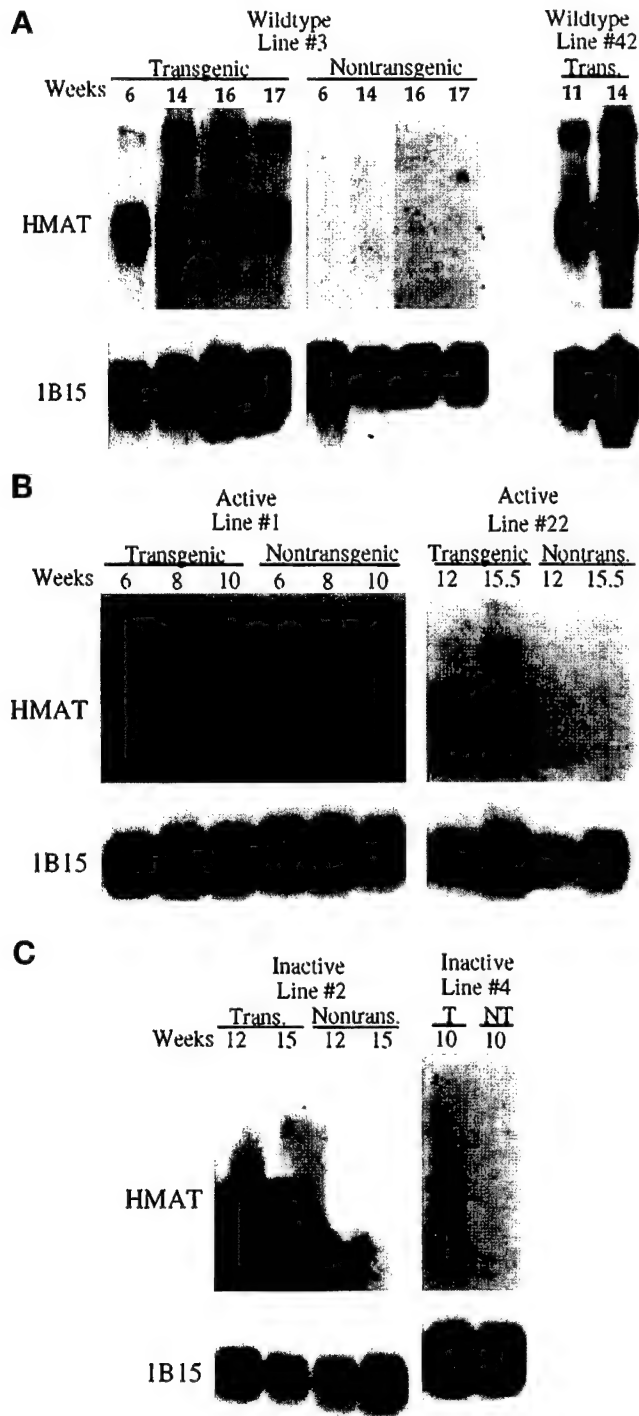


Figure 3. Expression of the human MAT transgene in developing mammary tissue. Northern analysis of poly A⁺ selected RNA (4 μ g) from nontransgenic and transgenic female mammary tissue at various weeks during mammary development. Blots were probed with a 1.1-kb ³²P-labeled human MAT cDNA probe (HMAT), and a cyclophilin (1B15) cDNA probe was used to control for RNA loading. (A) Expression of MAT transgene is present at relatively high levels in selected samples from lines 3 and 42 and absent in the nontransgenic littermates. (B) Line 1 and line 22 express the MMTV-



Figure 4. Localization of human MAT protein by immunohistochemistry. Representative sections from developing transgenic (A, B, and C) and nontransgenic (D) mammary glands were probed with an affinity-purified polyclonal antibody against a human MAT peptide. Staining of human MAT is in the cytoplasm of the mammary ducts from MMTV-MAT line 3 (A) and to a lesser extent in MMTV-ActMAT line 22 (B) developing glands (arrows). No specific staining was detected in MMTV-InMAT line 2 transgenic (C) or nontransgenic (D) mammary glands. Photographs were taken using a 50 \times objective.

Consequences of MAT Overexpression in the Mammary Glands

Previous studies have indicated that the expression of the MMP STR-1 in mammary epithelial cells results in disruption of the basement membrane and subsequent

Figure 3 (cont). ActMAT transgene, while nontransgenic littermates do not express the transgene. (C) Isolation of intact human MAT mRNA from both MMTV-InMAT transgenic lines 2 and 4 was not possible after several attempts. Degraded human MAT mRNA as shown was consistently isolated in both lines of transgenic mammary glands.

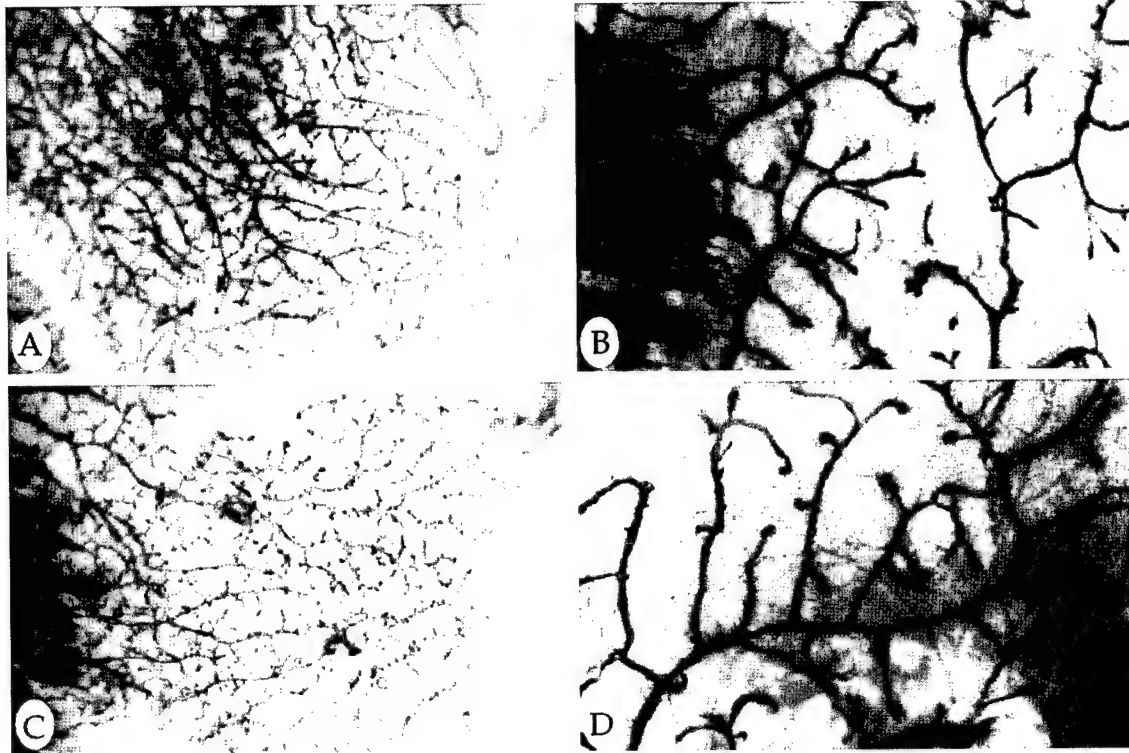


Figure 5. Morphological appearance of developing mammary glands. Iron hematoxylin-stained whole mounts of inguinal mammary glands from MMTV-MAT line 3 transgenic (A and B) and nontransgenic (C and D) female virgin animals. Glands were removed at 14 wk (A and C), 16 wk (B), and 12 wk (D) of mammary gland development. Photographs were taken using a 2.5 \times (A and C) and 6.4 \times (B and D) objective.

changes in the proliferative and apoptotic indices of these cells, as well as premature lobuloalveolar development and milk protein production in virgin female mice (Simpson *et al.*, 1994; Witty *et al.*, 1995b). STR-1 is normally expressed during murine mammary gland development; however, its expression is confined to stromal cells surrounding the developing ducts (Witty *et al.*, 1995b). We have shown that MAT is endogenously expressed, albeit at very low levels, in the adult murine mammary gland by reverse transcriptase-PCR (Wilson *et al.*, 1995). Although we have been unable to localize endogenous MAT expression in this tissue by *in situ* hybridization or immunohistochemistry, studies with human mammary glands demonstrate that MAT mRNA and protein are expressed in mammary epithelial cells (Saarialho-Kere *et al.*, 1995; Heppner *et al.*, 1996). Examination of other murine tissues also suggests that MAT is primarily expressed in glandular epithelial cells (Wilson *et al.*, 1995). Therefore, we were interested in investigating the effects of overexpressing the epithelium-specific MMP MAT in the epithelial cells of developing murine mammary glands and comparing the effect to previous results with STR-1 overexpression.

The ductal tree of developing mammary glands in nontransgenic and MMTV-MAT transgenic mice was

examined by whole mount tissue preparation. During mammary gland development, which begins at approximately 5 wk of age and following the onset of estrogen production, the mammary end buds grow outward from the nipple to fill the entire fat pad with a highly branched network of epithelial cells (Snedeker *et al.*, 1991 and references therein). There was no apparent morphological difference in the mammary ductal tree during development, pregnancy, lactation, or involution in MMTV-MAT (Figure 5A and B, and unpublished results) when compared with nontransgenic littermate controls (Figure 5C and D, and unpublished results). We also observed no difference in mammary gland morphology in the MMTV-ActMAT and MMTV-InMAT animals.

Although the MMTV-MAT glands display normal morphology, we examined them for subtle changes in differentiation, proliferation, or apoptosis that may have occurred in response to MMTV-MAT. Production of the milk proteins in the casein family is normally restricted to differentiated mammary epithelial cells during late pregnancy and lactation. However, using an antibody specific for mixed caseins, milk proteins were detected in all virgin MAT transgenic animals previously shown to express the MAT transgene by Northern analysis or MAT protein by immu-

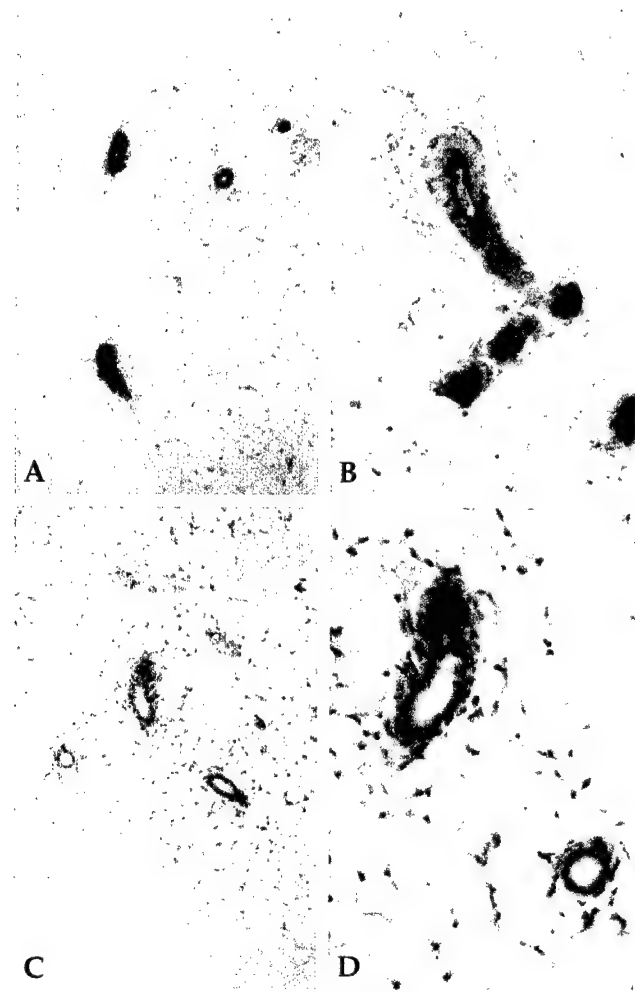


Figure 6. Casein expression in virgin MMTV-MAT mammary glands. MMTV-MAT line 3 transgenic mammary glands (A and B) at 6 wk of age were positive for β -casein expression using immunohistochemistry, while nontransgenic control mammary glands (C and D) were negative. Panels A and C were taken using a 16 \times objective, and panels B and D were taken using a 32 \times objective.

nohistochemistry (Figure 6A and B). No casein protein was detected in age-matched nontransgenic control mammary glands (Figure 6C and D). These results suggest that there is aberrant differentiation of mammary epithelial cells as a result of the transgene expression, although there are no accompanying morphological changes resembling lobuloalveolar development. The lack of morphological changes is consistent with our inability to detect differences in the number of proliferative or apoptotic cells in the MMTV-MAT mammary glands compared with age-matched, nontransgenic controls. We observed no significant difference in the number or location of proliferating cells as determined by immunoreactivity with the cell cycle marker proliferating cell nuclear antigen, or apoptotic cells as determined by the number of cells

with excessive nuclear DNA fragmentation (TUNEL assay; unpublished results).

Localization of MAT Transgene Expression in the Male Reproductive Tract

The MMTV-LTR targets expression of a reporter gene to the male reproductive tract (Choi *et al.*, 1987; Ross *et al.*, 1990). Several transgenic animals previously generated using this promoter have reported transgene expression in the testis and epididymis (Witty *et al.*, 1995b; Matsui *et al.*, 1990). In agreement with this, we found that the MAT transgene was expressed in both the testis and epididymis of MMTV-MAT transgenic mice. More specifically, the human MAT mRNA was localized by in situ hybridization to the primary spermatocytes of the transgenic testis (Figure 7A and B). In contrast, the human MAT protein localizes by immunohistochemistry on frozen sections of the testis to the interstitial space surrounding the seminiferous tubules (Figure 7C and D). In the transgenic epididymis, the human MAT protein was also localized by immunohistochemistry to the epithelial ducts of the initial segment of the epididymis (Figure 8, A and B). Endogenous murine MAT protein has been previously shown to be expressed in the epithelial cells lining the efferent ducts (Wilson *et al.*, 1995). The antibody that we have utilized for these studies is specific for human MAT as shown by the inability of this antibody to detect endogenous mouse MAT protein in the efferent ducts (Figure 8, A and B, and unpublished results). In addition, the human MAT antibody detects the transgene product in the transgenic testis (Figure 7C and D) whereas the antibody specific for mouse MAT protein does not show specific staining in transgenic testis (unpublished results).

Consequences of MAT Overexpression in the Male Reproductive Tract

When breeding the MMTV-MAT male transgenics, we noted that one of the male founder animals was infertile. In addition, other male founders and their offspring also demonstrated reduced fertility, producing few litters with only one to three pups. These same male transgenic animals eventually became infertile at approximately 6 mo of age. We therefore analyzed several male transgenic gonads to address the cause of the observed reproductive defect. Hematoxylin and eosin-stained sections of testis from 8-mo-old or older transgenic males show extreme disorganization of seminiferous tubule morphology (Figure 7F, for example) compared with an age-matched nontransgenic controls (Figure 7E). In addition, an absence or reduced number of mature spermatozoa is noted in the seminiferous tubules of transgenic testis (Figure 7F) compared with the abundant presence of spermatozoa in the lumen of nontransgenic testis (Figure 7E). Less

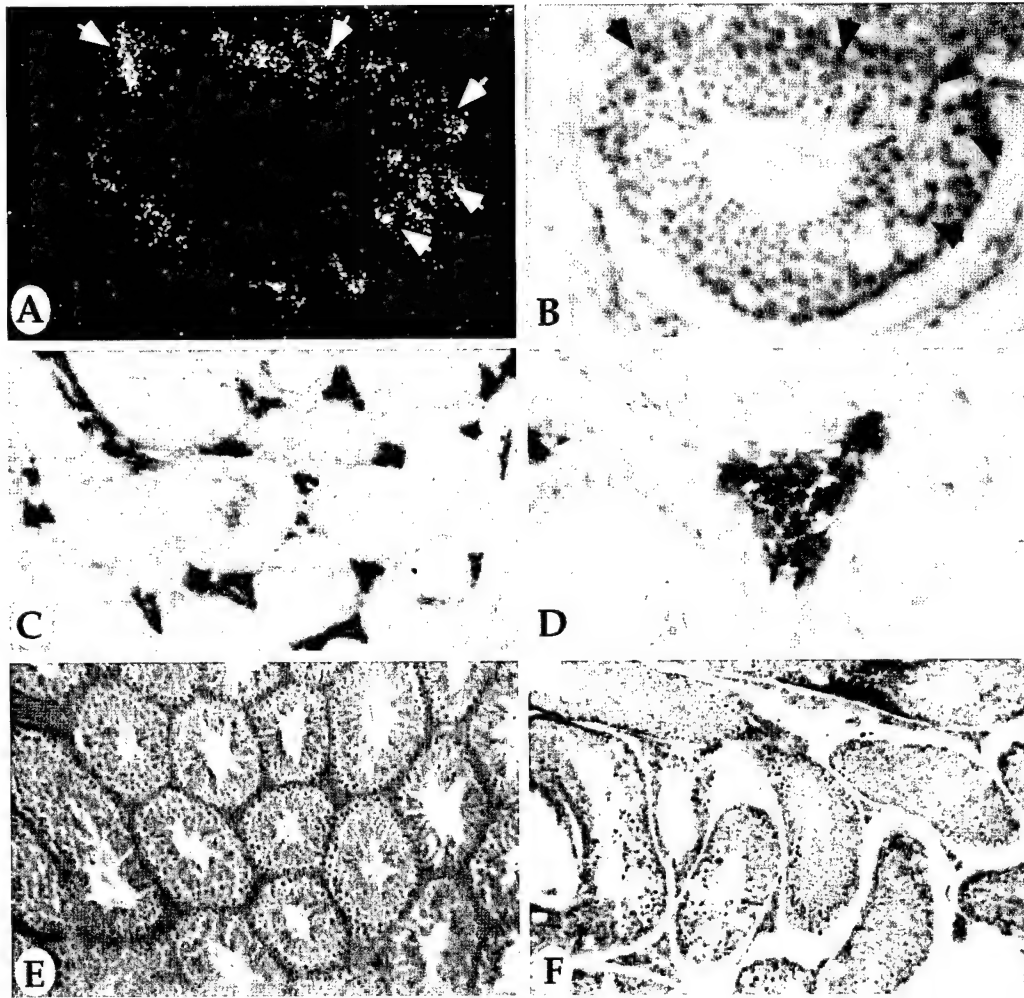


Figure 7. Expression and consequence of human MAT in the testis. Sections from the testis of a young (4- to 6-mo-old) MMTV-MAT line 3 male were hybridized with an antisense ^{35}S -labeled human MAT probe (A, dark-field and B, light-field; 40 \times objective). Hybridization was localized to the primary spermatocytes (arrows) in the testis (A and B). Frozen sections of the testis from a young (4- to 6-mo-old) MMTV-MAT line 3 transgenic male were probed with a polyclonal antibody specific to human MAT. MAT transgene product is localized to the interstitial space surrounding the seminiferous tubules as can be seen at low power (C; 12.5 \times objective) and at higher power (D, 50 \times objective). Hematoxylin and eosin staining of sections obtained from an aged (8- to 9-mo-old) normal (E) or MMTV-MAT line 3 transgenic (F) adult testis (both taken using a 12.5 \times objective).

severe morphological disruption was also observed in younger males. The MMTV-MAT transgenic epididymis demonstrated a lack of mature sperm production (Figure 8D), and the abnormal presence of sloughed undifferentiated germ cells was observed at higher magnification (Figure 8F), compared with age-matched nontransgenic controls (Figure 8, C and E). The morphology of the epididymis, and specifically the epithelial cells of the initial segment, appear normal (Figure 8), indicating that this is a local response and not a general effect on the male reproductive tract. Consistent with this observation, circulating androgen levels in aged transgenic male animals was not significantly different from age-matched nontransgenic controls (unpublished results). These histological data

support our initial observations of decreased fertility in the MMTV-MAT male transgenic animals. To our knowledge, perturbation of male reproductive function by MMP expression has not been previously observed.

Expression of the MAT Transgene in Other Organs

The MMTV-LTR is known to be expressed in the epithelial cells of the salivary glands, lungs, kidneys, and lymphoid cells of the spleen and thymus in addition to the tissues discussed above (Ross *et al.*, 1990). We detected human MAT mRNA in the adult brain, salivary glands, lung, spleen, and thymus of MMTV-MAT transgenic mice, while the female reproductive

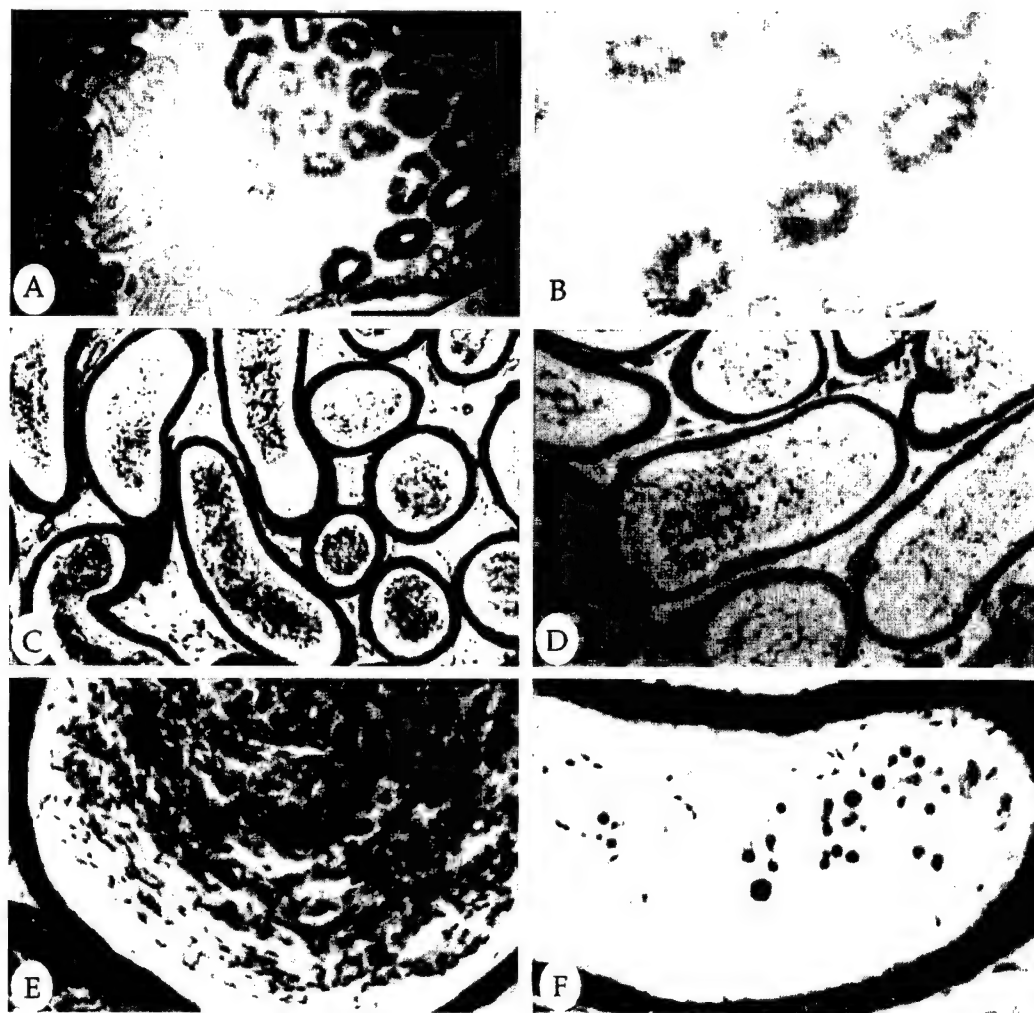


Figure 8. Expression and consequence of human MAT in the epididymis. Frozen sections of the epididymis from a MMTV-MAT line 3 transgenic male were probed with a polyclonal antibody specific to human MAT. The protein is localized to the epithelial ducts of the uppermost region of the epididymis, the initial segment (in. seg.), as can be seen at low power (A; 6.25 \times objective; distal caput, dis. cap.; efferent ducts, e. d.) and high power (B; 12.5 \times objective). Hematoxylin and eosin staining of normal (C and E) or MMTV-MAT line 3 transgenic (D and F) adult corpus epididymis. Pictures taken using a 16 \times (C and D) and 50 \times (E and F) objective.

tract and liver contained no detectable human MAT RNA (unpublished results). Although these additional organs expressed MAT, there were no observable functional or morphological changes in these tissues as a result of transgene expression.

DISCUSSION

Our goal in this study was to generate transgenic animals that overexpress three separate forms of human MAT in the reproductive tissues. To this end, we first developed wild-type, active, and inactive human MAT constructs and examined the ability of these constructs to produce functional MAT protein in an *in vitro* system. We have shown that full-length wild-type human MAT cDNA under the control of the

MMTV-LTR promoter/enhancer was capable of producing full-length pro-MAT that was efficiently converted to active MAT after exposure to an exogenous activator. A valine-to-glycine substitution in the highly conserved prodomain sequence PRCGVDPV of MAT encoded a protein with an increased tendency to spontaneously generate active protein, similar to that previously observed with STR-1 (Sanchez-Lopez *et al.*, 1988; Park *et al.*, 1991). In addition, a glutamic acid-to-glutamine substitution in the conserved zinc-binding sequence of MAT resulted in a catalytically inactive protein, as observed for STR-1 (Sanchez-Lopez *et al.*, 1988; Park *et al.*, 1991). Although we could detect MAT protein produced by these constructs in the conditioned medium of cultured breast carcinoma cells,

mutations of the MAT cDNA significantly decreased the efficiency with which MAT protein was produced in the transgenic mammary gland *in vivo*. The relative mRNA and protein levels of MAT were reduced in animals expressing the active MAT construct, and we observed no intact mRNA and no immunostaining for MAT protein in animals containing the inactive MAT transgene. The effect of the inactivating mutation on mRNA stability is unusual, although nonsense mutations have been shown to result in increased mRNA decay in several different organisms (Kessler and Chasin, 1996 and references therein). It is presumed that this effect occurs in the cultured cells as well, but that sufficient mRNA is present to allow translation and accumulation of protein in the culture medium. A reduction in the efficiency of protein production was noted previously after mutation of similar sequences in the STR-1 protein (Sanchez-Lopez *et al.*, 1988; Park *et al.*, 1991). It was speculated that this was due to the presence of active enzyme in an intracellular compartment resulting in premature auto-degradation (Park *et al.*, 1991), or possibly a conformational change that may have altered the secretory pathway of the mutant protein. Similar effects are likely to have occurred with the mutated MAT protein *in vitro* and *in vivo*, resulting in a reduction in protein levels. The absence of detectable inactive MAT protein *in vivo* may reflect a protein turnover system that is not operable in cultured cells or is reliant on other cell types.

The expression of wild-type MAT results in an altered phenotype in the mammary gland and male reproductive tract compared with nontransgenic controls. We assume this is the result of degradation of MAT substrates, although we were unable to definitively attribute the effect to MAT-induced proteolysis since the inactive MAT transgenic mice expressed no detectable MAT protein. However, since MAT is the "minimal domain MMP," it is unlikely to possess biological activities other than proteolysis. Since phenotypic alterations were observed in the mammary gland and testis, wild-type MAT is apparently activated in the tissues. Similar endogenous activation of interstitial collagenase was assumed in transgenic mice expressing a human MMP-1 genomic fragment under the control of the haptoglobin promoter (D'Armiento *et al.*, 1992). MMPs such as MAT are activated by the "cysteine switch" mechanism, in which the cysteine in the conserved PRCGVDFV sequence in the pro domain becomes dissociated from the catalytic Zn, resulting in a conformation change and autoproteolysis of the pro domain (Van Wart and Birkedal-Hansen, 1990). The activation of latent MMPs is affected by a variety of natural molecules. For example, plasmin activates most MMPs by cleaving once in the pro domain, producing an unstable intermedi-

ate form of the enzyme, which then autoproteolyses to produce a fully active enzyme (Birkedal-Hansen *et al.*, 1993 for review). Other enzymes, such as cathepsin G, neutrophil elastase, trypsin, chymotrypsin, and plasma kallikrein, have also been shown to activate latent MMPs by similar mechanisms (Eeckhout and Vaes, 1977; Grant *et al.*, 1987; Okada and Nakanishi, 1989; Nagase *et al.*, 1990; Saari *et al.*, 1990). In addition to proteases, oxygen radicals are also potential activators of MMPs due to their ability to disrupt the cysteine switch (Burkhardt *et al.*, 1986; Rajagopalan *et al.*, 1996). At this time, the endogenous activator of MAT is unidentified, but it is interesting that phenotypic alterations are observed in some, but not all, tissues that express the wild-type MAT transgene. A more thorough examination of tissues in the MMTV-Act-MAT mice may provide insights as to whether the absence of phenotypic differences is due to the lack of an effect of MAT in these organs or the deficiency of an endogenous activator of latent MAT.

The Effect of MAT Overexpression on Mammary Gland Development

The developing murine mammary gland has provided an excellent model system to examine the role of MMPs in a remodeling tissue. The expression patterns of MMPs suggest that they play an important role in the dramatic morphological and functional changes that take place in the mammary gland during ductal development. STR-1 and GEL A in particular are expressed in the developing mouse mammary gland as well as during the involution process (Talhouk *et al.*, 1992; Sympson *et al.*, 1994; Witty *et al.*, 1995b). MAT mRNA, in contrast, is expressed at low levels in the murine mammary gland (Wilson *et al.*, 1995), but is found in abundant levels in human mammary epithelium from reduction mammoplasties (Saarialho-Kere *et al.*, 1995; Heppner *et al.*, 1996). Since the function of MAT in human mammary epithelium is unknown, recapitulation of MAT expression in the murine mammary gland provides a system in which to address this question.

Overexpression of human MAT protein had no effect on the general morphological development of the mammary ductal tree, but induced the ectopic expression of a pregnancy-associated protein, β -casein, in developing virgin transgenic mammary glands. In contrast, the MMTV-STR-1 (Witty *et al.*, 1995b) and WAP-STR-1 (Sympson *et al.*, 1994) transgenic animals express β -casein mRNA, but not protein, display the morphological features of precocious lobuloalveolar development, and demonstrate increased proliferation and apoptotic indices (Boudreau *et al.*, 1995; Witty *et al.*, 1995b). There are several potential explanations for these differences. Experimental variation, such as differences in the integration sites, expression levels, and

genetic backgrounds of the mice, may be contributing factors, although the phenotypes were observed in several independent lines of mice in all cases. STR-1 contains a hemopexin/vitronectin-like domain that is absent in MAT, and may confer additional activities or alter substrate specificity *in vivo* resulting in the observed phenotypic differences. The abnormal tissue-type expression of STR-1 in glandular epithelial cells, as opposed to the normal expression in stromal fibroblast-like cells surrounding the developing ducts (Witty *et al.*, 1995b), may also account for the more profound cellular alterations in these mice compared with MAT transgenic animals. In addition, the differential endogenous expression levels of STR-1 and MAT in the mammary gland suggest that these MMPs may have distinct roles during mammary development. The low endogenous expression levels of MAT (Wilson *et al.*, 1995) implies that this particular MMP plays a minor role in mammary development compared with the abundantly expressed STR-1 (Witty *et al.*, 1995b), which may explain the less dramatic consequences of MAT overexpression. Although the morphological features of lobuloalveolar development were not observed in the MMTV-MAT transgenic mice, they displayed features of lactational differentiation by the production of β -casein protein in virgin transgenic mammary glands. This implies that β -casein expression can be dissociated from the morphological changes and may be directly related to alterations in the integrity of the basement membrane of mammary epithelial cells.

MMTV-MAT Expression in Male Reproductive Tract Induces Infertility

An unexpected consequence of generating MAT transgenic animals under the control of the MMTV promoter/enhancer was the development of abnormalities of the male reproductive tract, since this phenotype was not observed in the MMTV-STR-1 mice (Witty *et al.*, 1995b). In the testis, spermatozoa normally develop within the seminiferous tubules in close association with the Sertoli cells, while androgens are synthesized between the tubules in the Leydig cells (reviewed in Johnson and Everitt, 1995). These two compartments are separated by structural and physiological barriers that develop during puberty before the initiation of spermatogenesis. The barriers consist of gap and tight junctional complexes that completely encircle each Sertoli cell, linking it to the next adjacent cell. A few molecules may traverse these junctional complexes and penetrate into the basal compartment of the tubules from the surrounding interstitium, usually as the result of selective transport. Ions and proteins not only flow from the Leydig cells into the tubules, but proteins such as androgen-binding protein, testicular transferrin, and sulfated glycoproteins 1 and 2 move

from the intratubular compartment out into the interstitial area surrounding the tubules (Griswold, 1988; Gunsalus and Bardin, 1991). Similar to these proteins, the mRNA and protein localization patterns of the MAT transgene suggest that MAT protein is produced by the germ cells of the seminiferous tubules and selectively transported into the interstitial space surrounding the Leydig cells. The functional consequence of this is to disrupt sperm production, as evidenced by the absence of mature spermatozoa in the epididymis of these transgenic male animals. This is presumably due to the degradation of the cellular barriers between the interstitium and seminiferous tubules and subsequent loss of tissue architecture. The decrease in sperm production is not a direct result of a loss of Leydig cell function, as demonstrated by the maintenance of normal testosterone levels in transgenic animals. The degradative effects of MAT seem to be gradual, suggesting that a threshold of excess enzyme needs to be reached before damaging effects occur, or that the destructive effects are cumulative. The absence of mature spermatozoa in the transgenic epididymis could also be caused by the overexpression of MAT in the initial segment of the epididymis. Overexpression of the MAT transgene in the epididymis may have specific effects on sperm maturation by disrupting the spatial or temporal cleavage of specific substrates or may have more nonspecific effects caused by excessive degradation of proteins in these organs.

High levels of endogenous MAT expression have been localized to the efferent ducts while low levels of endogenous MAT hybridization were also observed in the proximal area of the initial segment of the epididymis and in the cauda, where mature fully differentiated sperm accumulate in the lumen (Wilson *et al.*, 1995). Very little is known about the endogenous expression of other metalloproteinases and their inhibitors in the male reproductive tract. GEL A is expressed by cultured rat Sertoli cells (Sang *et al.*, 1990a,b), and TIMP-1 and TIMP-2 have been detected in the Sertoli cells of maturing rats (Ulissee *et al.*, 1994). In addition, precursor regions in the α - and β -subunits of the fertilin complex or PH-30, a sperm surface protein that has been implicated in sperm-egg fusion, contain metalloproteinase domains that align with those found in snake venom proteins (Wolfsberg *et al.*, 1993). The function of MAT and other MMPs in the male reproductive system is not known. However, the specific tissue expression pattern of endogenous MAT is suggestive of a role in sperm maturation, possibly by proteolytically processing sperm antigens. MAT-deficient and STR-1-deficient mice display no obvious defects in male fertility (Wilson *et al.*, 1997 and our unpublished observations). In earlier studies we have observed up-regulation of STR-1 and STR-2 in the involuting uterus of MAT-deficient mice, and a similar

apparent compensatory mechanism in STR-1-deficient mice (Rudolph-Owen *et al.*, 1997). These data suggest that there is strong selective pressure for MMP activity in reproductive processes, which we speculate may also include the male reproductive tract.

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REVIEW

Changing Views of the Role of Matrix Metalloproteinases in Metastasis

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Metastatic spread of cancer continues to be the greatest barrier to cancer cure. Understanding the molecular mechanisms of metastasis is crucial for the design and effective use of novel therapeutic strategies to combat metastases. One class of molecules that has been repeatedly implicated in metastasis is the matrix metalloproteinases (MMPs). In this review, we re-examine the evidence that MMPs are associated with metastasis and that they make a functional contribution to the process. Initially, it was believed that the major role of MMPs in metastasis was to facilitate the breakdown of physical barriers to metastasis, thus promoting invasion and entry into and out of blood or lymphatic vessels (intravasation, extravasation). However, recent evidence suggests that MMPs may have a more complex role in metastasis and that they may make important contributions at other steps in the metastatic process. Studies using intravital videomicroscopy, as well as experiments in which levels of MMPs or their inhibitors (tissue inhibitors of metalloproteinases [TIMPs]) are manipulated genetically or pharmacologically, suggest that MMPs are key regulators of growth of tumors, at both primary and metastatic sites. On the basis of this evidence, a new view of the functional role of MMPs in metastasis is presented, which suggests that MMPs are important in creating and maintaining an environment that supports the initiation and maintenance of growth of primary and metastatic tumors. Further clarification of the mechanisms by which MMPs regulate growth of primary and metastatic tumors will be important in the development of novel therapeutic strategies against metastases. [*J Natl Cancer Inst* 1997;89:1260-70]

Considerable research has been directed toward understanding both the steps involved in metastatic spread of cancer cells and the underlying molecular mechanisms. Understanding the molecular basis of metastasis is crucial for the development and appropriate clinical use of novel therapeutics directed at prevention of metastasis and its consequences to the patient. Here we will discuss one class of molecules, the matrix metalloproteinases (MMPs), enzymes that have been repeatedly implicated in metastasis. Our goals in this review are to re-examine the evidence that MMPs are associated with the metastatic phenotype, that they contribute functionally to metastasis, and how they do so. We will not attempt to review the full literature on these topics, since it is extensive and many recent reviews have sum-

marized and critically evaluated much of this literature (1-7). Instead, we will focus conceptually on changing ideas about the nature of the roles of MMPs in the metastatic process, based primarily on recent studies that suggest that their major contribution may be somewhat different and more complex than previously assumed. Clarification of the molecular nature and timing of the contributions of MMPs to metastasis are important in part because MMPs are viewed as an appropriate target for antimetastasis therapies, and use of this therapeutic strategy will be maximized if the roles of MMPs in metastasis, both temporally and spatially, are well understood.

Overview of the Metastatic Process

Metastasis is the spread of cancer from a primary tumor to distant sites of the body and is a defining feature of cancer (8,9). Metastasis is defined by end points, i.e., metastatic lesions detected in specific organs distant from a primary tumor, while steps by which metastases form have often been inferred rather than directly observed. Some experimental and clinical evidence supports some of these steps, but the internal nature of the process has prevented it from being fully understood. Sequential steps in the process (Fig. 1) [reviewed in (8-12)] are believed to include the following: escape of cells from the primary tumor, intravasation (entry of cells into the lymphatic or blood circulation), survival and transport in the circulation, arrest in distant organs, extravasation (escape of cells from the circulation), and growth of cells to form secondary tumors in the new organ environment. Angiogenesis, the recruitment of new blood vessels, is required for the primary and metastatic tumors to grow beyond minimal size, and evasion of immune destruction is necessary at various steps throughout the process. The end point, formation of detectable metastatic lesions, thus can be prevented by interruption at any one or more of these steps.

Metastasis is known to be an inefficient process, from both clinical observations and experimental studies [(13-17); re-

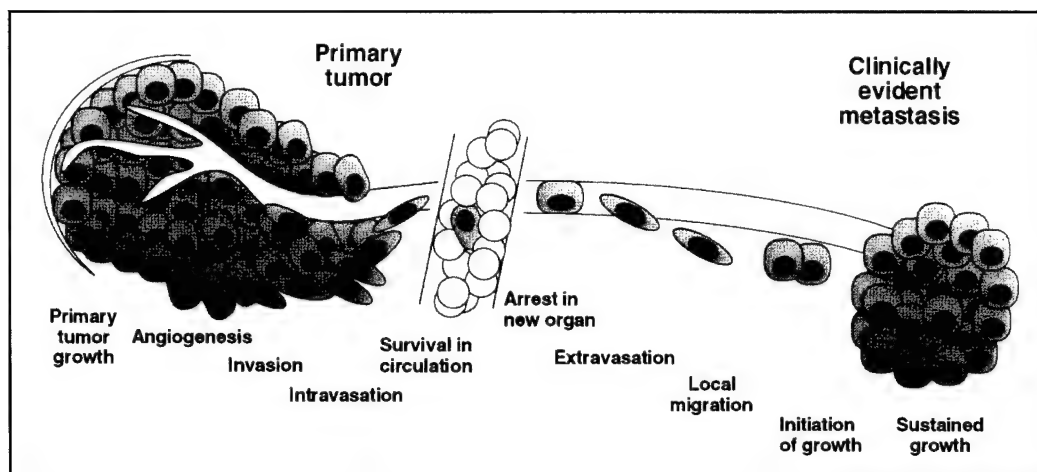
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Fig. 1. Metastatic process. Tumor cells are believed to proceed through the sequential steps indicated to form clinically detectable metastases.



viewed in (18)]. Large numbers of cells can be shed into the circulation from a primary tumor, and yet not all of these cells will form metastases. When cells are injected into the circulation of experimental animals, only a small fraction of the cells will succeed in forming metastases. Furthermore, experimental studies (19–21) indicate that individual metastases are likely clonal in origin. Thus, the majority of cells that successfully escape from a primary tumor will not complete all of the steps necessary to give rise to metastatic tumors. Steps that have been considered to be major contributors to this inefficiency, and thus rate limiting for metastasis, include cell survival in, and escape from, the circulation. Relatively few cells arriving in a target organ were believed to survive initial arrest, due to hemodynamic destruction, and of those that did survive, few were believed to succeed in extravasating. These views of the major rate-limiting steps in metastasis have been questioned by recent studies using *in vivo* videomicroscopy to directly monitor the fate of cells during the metastatic process, as discussed below.

Metastasis is the final stage in tumor progression from a normal cell to a fully malignant cell. Considerable progress has been made in identifying molecular changes that accompany, and may be responsible for, the clinical, pathologic, and cytogenetic changes that occur during the progression of specific cancers (22,23). The best-developed example is the characterization of molecular progression in colon cancer, in which specific changes (e.g., loss of tumor-suppressor genes and mutation of oncogenes) are preferentially associated with specific stages of progression (24,25). However, the final stage in tumor progression to a metastatic phenotype has eluded characterization at a molecular genetic level, in colon or other cancers. Initially, there was hope that a single metastasis-specific gene could be identified to be responsible for conversion to a metastatic phenotype. Early DNA transfection studies indicated that some cells could be converted to a metastatic phenotype by this strategy, suggesting that metastatic ability could have a genetic basis [reviewed in (26)]. In addition, transfection with a variety of oncogenes (e.g., *ras* and *src*) could produce metastatic cells [reviewed in (27)]. These studies indicated that a variety of downstream, oncogene-regulated genes could functionally contribute to metastatic behavior of the cells. For example, metastatic H-*ras*-transfected NIH 3T3 cells had increased levels of a variety of gene products, including proteinases and adhesive proteins, ac-

companied by decreases in other gene products, including proteinase inhibitors (28). Loss of tumor-suppressor gene function also has been implicated in the conversion to metastatic ability in specific tumor types, although none is likely to be universally implicated in all tumor types [e.g., *nm23* (29); *KAI1* (30), *KiSS-1* (31); reviewed in (32)].

Thus, phenotypically there appear to be cellular abilities necessary for metastasis in many tumor types, while genotypically there does not seem to be a single master “metastasis gene” that regulates these properties in all tumors. It appears more likely that regulation of expression of genes that contribute functionally to metastasis can occur in a tissue-specific manner, with different regulatory genes (e.g., oncogenes and tumor-suppressor genes) inducing the multiple aspects of the metastatic phenotype in specific tumors. Included among the required traits is sufficient proteolytic capacity to complete all the steps in metastasis. MMPs and their inhibitors have been repeatedly implicated in this context. We next will summarize this family of enzymes and their inhibitors and then will consider their relationship to the process of metastasis.

Metalloproteinases and Their Inhibitors

MMPs are a family of secreted or transmembrane proteins that are capable of digesting extracellular matrix and basement membrane components under physiologic conditions. Currently, 16 family members have been identified (Fig. 2). They share a catalytic domain with the HEXGH motif responsible for ligating zinc, which is essential for catalytic function. MMPs are also characterized by a distinctive PRGVPD sequence in the pro domain that is responsible for maintaining latency in the zymogens. MMP family members differ from each other structurally by the presence or absence of additional domains that contribute to activities, such as substrate specificity, inhibitor binding, matrix binding, and cell-surface localization [reviewed in (6,33)]. There are three major subgroups of MMPs, identified by their substrate preferences: collagenases degrade fibrillar collagen, stromelysins prefer proteoglycans and glycoproteins as substrates, and gelatinases are particularly potent in degradation of nonfibrillar and denatured collagens (gelatin).

MMP activity is highly regulated at many levels. The messenger RNA (mRNA) for most family members is transcriptionally regulated by biologically active agents, such as growth fac-

Fig. 2. Matrix metalloproteinase (MMP) family. Subgroups are arranged by domain structure and separated by a dashed line. Within the largest, hemopexin-domain subgroup, family members with some distinct but subtle structural features are separated by a dotted line. This table is adapted from Powell and Matrisian (6) with the addition of information from references (123–127). MT = membrane type; PRE = leader sequence; PRO = prodomain; CAT = catalytic domain; H = hinge domain; HEM = hemopexin-like domain; F = furin consensus site; FN = fibronectin-like domain; C = collagen-like domain; TM = transmembrane domain; and ND = not determined.

MMP	DOMAIN STRUCTURE	MAJOR SUBSTRATES
Matrilysin (MMP-7, pump-1) (EC 3.4.24.23)	PRE PRO CAT	Proteoglycans, ECM glycoproteins, IV collagen, gelatins, elastin
Interstitial Collagenase (MMP-1, EC 3.4.24.7)	PRE PRO CAT H HEM	Fibrillar Collagens
Neutrophil Collagenase (MMP-8, EC 3.4.24.34)	PRE PRO CAT H HEM	Fibrillar Collagens
Collagenase-3 (MMP-13)	PRE PRO CAT H HEM	Fibrillar Collagen
Stromelysin-1 (MMP-3, transin, EC 3.4.24.17)	PRE PRO CAT H HEM	Proteoglycans, ECM glycoproteins, IV collagen, gelatins
Stromelysin-2 (MMP-10, transin-2, EC 3.4.24.22)	PRE PRO CAT H HEM	Proteoglycans, ECM glycoproteins, IV collagen, gelatins
Metalloelastase (MMP-12, EC 3.4.24.65)	PRE PRO CAT H HEM	Elastin
MMP-18	PRE PRO CAT H HEM	N.D.
MMP-19	PRE PRO CAT H HEM	N.D.
Stromelysin-3 (MMP-11)	PRE PRO F CAT H HEM	Laminin and fibronectin (weakly)
MT1-MMP (MMP-14)	PRE PRO F CAT H HEM TM	Gelatinase A, fibrillar collagens, proteoglycans, ECM glycoproteins
MT2-MMP (MMP-15)	PRE PRO F CAT H HEM TM	N.D.
MT3-MMP (MMP-16)	PRE PRO F CAT H HEM TM	Gelatinase A
MT4-MMP (MMP-17)	PRE PRO F CAT H HEM TM	N.D.
Gelatinase A (MMP-2, 72kD gelatinase, IV collagenase, EC 3.4.24.24)	PRE PRO CAT FN CAT H HEM	Gelatins, collagen IV, collagen I
Gelatinase B (MMP-9, 92kDa gelatinase, IV collagenase, EC 3.4.24.35)	PRE PRO CAT FN CAT C H HEM	Gelatins, collagen IV, collagen V

tors, hormones, oncogenes, and tumor promoters. There is evidence for regulation at the level of mRNA stability, translational control, and storage in secretory granules for specific MMPs in specific cell types. In general, however, the protein is rapidly secreted in a latent form and requires extracellular activation. Proteinase cascades involving other MMPs as well as other enzyme classes have been implicated in MMP activation. Once the enzymes are active, they are susceptible to inhibition by the general serum proteinase inhibitor α 2-macroglobulin and by a family of specific tissue inhibitors, the tissue inhibitors of metalloproteinases (TIMPs). There are currently four members of the TIMP family that have in common their MMP inhibitory activity but differ in properties, such as expression patterns and association with latent MMPs (Table 1). TIMPs act to inhibit metalloproteinase activity by forming a complex with active MMPs and are believed to be specific for enzymes of this fam-

ily, although they do not distinguish effectively between individual family members.

MMPs were originally described as enzymes responsible for dissolution of the tadpole tail (34). Subsequent work focused on the association of these activities with systems characterized by dramatic connective tissue remodeling, such as uterine involution, wound healing, and joint destruction in arthritic conditions. The notion that MMPs are the major class of enzymes responsible for matrix degradation is supported not only by the "smoking gun" nature of their association with these processes but also by the observation that members of this family are the only enzymes known to denature and digest fibrillar collagens. More recently, experiments involving genetic manipulation of MMPs or their inhibitors and specific synthetic inhibitors provide additional support for the essential role of these enzymes in normal and pathologic matrix destruction.

Table 1. TIMP family*

	TIMP-1	TIMP-2	TIMP-3	TIMP-4
Molecular mass	28 kd	21 kd	24 kd	22 kd
Messenger RNA	0.9 kb	1.1/3.5 kb	4.5–5.0 kb	1.4 kb
Associated proteins	proGELB	proGELA	ECM	Not determined
Major sites of expression	Ovary, bone	Placenta	Kidney, brain	Heart

*Information presented in this table is reviewed in (33) and presented in (117–122).

Association of MMPs With Cancer

During metastasis, there are a series of collagen-containing structural barriers that cells must pass (*see* Fig. 1). Extracellular matrix and basement membrane barriers must be breached for cells to intravasate and extravasate. The basement membrane underlying endothelial cells presents, in many organs, a continuous collagen-containing structural barrier to completion of the metastatic process. Within tissue, at either primary or secondary tumor sites, extracellular matrices appear to require degradation to permit tumor cell invasion and spread. By logical inference, metastatic cells require sufficient degradative enzymatic capacity to break down these proteinaceous structural barriers. Alternatively, some of the required proteolytic activity may be derived from tumor-associated host tissues, including adjacent stromal tissue and tumor-infiltrating immune cells. Support for a requirement for enhanced proteolytic function associated with cancer comes from pathologic studies of tumors, in which defects in basement membranes adjacent to tumors are commonly associated with malignant but not benign tumors (35).

MMPs have been associated with the malignant phenotype for several decades [early reviews in (36–40)]. Several studies (41–43) presented evidence that malignant tumors contained proteolytic activity capable of degrading collagen *in vitro*. With the advent of more sophisticated biochemical and molecular biologic techniques, it became possible to identify individual proteases responsible for the activities detected in tumor cells. Proteases of all five major classes (i.e., serine, aspartic, cysteine, threonine, and metalloproteinases) have been linked with the malignant phenotype (44,45). From early work of Liotta et al. (41,42) and Tryggvason et al. (43), interest was focused on type IV collagenase, the enzyme responsible for degradation of type IV collagen, a major structural protein in basement membrane. The enzymes responsible for this activity are now recognized to be either gelatinase A (72 kd type IV collagenase) or gelatinase B (92 kd type IV collagenase). The first member of the MMP family to be cloned was transin, the rat homologue of stromelysin-1, which was identified as an oncogene and growth factor-inducible gene (46). Subsequent work identified the product of this complementary DNA (cDNA) as a protease that was overexpressed in malignant mouse skin tumors (47) and was related to the prototypic member of the MMP family, interstitial collagenase (48,49). Since that time, extensive literature demonstrating the association of MMP family members and tumor progression has developed [reviewed in (5,50)]. Several generalizations can be made: 1) The number of different MMP family members that can be detected tends to increase with progression of the tumor, 2) the relative levels of any individual MMP family members tend to increase with increasing tumor stage, and 3) MMPs can be made by either tumor cells themselves or, quite commonly, as a host response to the tumor. The expression pattern of MMPs, therefore, supports a role for these enzymes in later stages of tumor progression. MMPs are found most abundantly in tumors in which the basement membrane is breached and there is evidence for local invasion and distant metastases. In this review, we are considering the role of MMPs in tumor progression, but it should be remembered that they are not the only proteolytic contributors to this process, and interactions

between members of other classes of proteolytic enzymes provide additional levels of complexity and regulation (4,40,45).

Members of the TIMP family have also been associated with cancer. The literature on expression of TIMPs and MMPs in tumors has been thoroughly reviewed recently by Denhardt (7) and will not be considered further in detail here. An important point that must be made, however, is that the simplistic expectation that malignant tumors would have increased MMP expression accompanied by decreased TIMP expression is often not met. In several cases, malignant tumors have been shown to have increased rather than decreased TIMP levels [e.g., (51)]. Furthermore, the tissue localization of both specific MMPs and TIMPs in and around a tumor can be complex, with variable expression within the tumor versus adjacent stromal cells [reviewed in (7)]. Tumor localization studies can give only a snapshot at one point in time, and there are difficulties in interpreting these studies: for example, is overexpression of a particular enzyme or inhibitor an indication of a functional role for it in the malignant process or a sign of (effective or ineffective?) host response? One promising approach to address this question involves the use of transgenic or knockout mice to address the effects of altered host levels of specific MMPs or TIMPs [reviewed in (52)]. The localization and interplay between proteases and their inhibitors *in vivo* is complex and as yet poorly understood.

Evidence for a Functional Role for MMPs and Their Inhibitors in Metastasis

Evidence that MMPs play a functional role in metastasis came originally from experiments with recombinant or genetically manipulated levels of TIMP-1. Schultz et al. (53) first showed that an intraperitoneal injection of recombinant TIMP-1 reduced lung colonization of intravenously injected B16F10 melanoma cells. A reduction in TIMP-1 levels by antisense RNA in mouse fibroblasts resulted in formation of metastatic tumors in nude mice (54). Subsequent studies (55–58) using recombinant or transfected TIMP-1 or TIMP-2 in experimental and spontaneous metastasis assays further suggested that MMPs could play a causal role in metastasis. Assuming that the primary activity of TIMP in these assays is inhibition of MMP activity, these results provide strong support for a role for MMPs in the establishment of metastatic lesions.

Studies with synthetic MMP inhibitors further support a requirement for MMP activity in the establishment of metastatic foci. These low-molecular-weight compounds are unlikely to have complicating activities distinct from inhibition of metalloproteinase activity, and their specificity appears to be restricted to enzymes closely related to matrix metalloproteinases. The British Biotech inhibitor batimastat (BB-94) was shown to reduce metastasis of melanoma, mammary carcinoma, and colorectal tumor cells in experimental metastasis assays (59–61) and of human colon (62) and breast (63) tumor cells injected orthotopically in nude mice. Other broad-spectrum MMP inhibitors have shown similar results in lung colonization assays (64,65). In addition, combination therapy with a gelatinase A-specific inhibitor and cytotoxic agents reduced invasion and metastasis of subcutaneously injected Lewis lung carcinomas (66).

Finally, there is evidence for a role for specific MMP family

members in tumor cell invasion and metastasis. Bernhard et al. (67) have demonstrated that gelatinase B expression is strongly associated with the metastatic ability of rat embryo fibroblasts and that its overexpression results in increased metastatic potential following injection into nude mice (68), while ribozyme inhibition of this enzyme decreases lung colonization (69). Transfection of a gelatinase A cDNA in a bladder cancer cell line increased the area of lung metastases (70), and MT1-MMP overexpression enhanced the survival of mouse lung carcinoma cells in the lungs of intravenously injected mice (71). A role for the MMP matrilysin in tumor invasion was demonstrated by transfection into human prostate cells and measuring invasion into the diaphragm of immunodeficient mice (72).

The effect of MMP activity in spontaneous and experimental metastasis assays has been associated with the ability to degrade basement membrane and extracellular matrix components, thus facilitating invasion through connective tissue and blood vessel walls. This view was supported by *in vitro* studies measuring invasion through amnion basement membrane, smooth muscle cell-generated basement membrane, or reconstituted basement membrane (Matrigel; Collaborative Research, Inc., Waltham, MA). An inhibition of *in vitro* invasion has been observed following the addition of recombinant or transfected TIMP-1 or TIMP-2 (40,53,73-76), and targeted disruption of the TIMP-1 gene resulted in an increase in *in vitro* invasion (77). Other studies (64,78) using synthetic inhibitors of metalloproteinases also support an effect of MMPs on the penetration of basement membranes. It should be noted, however, that following transfection of various MMP family members, positive effects on *in vitro* invasion have been documented (79), but there are also examples of a lack of a consistent effect in these assays (80,81). In addition, no change in *in vitro* invasion was detected in loss-of-function studies in which expression of stromelysin-3 (81) and matrilysin (80) were ablated by use of antisense technology. These results raise the possibility that at least some MMPs may affect steps in metastasis other than extravasation.

Evidence Suggesting That MMPs Play a Role in the Growth of Primary and Secondary Tumors

As outlined above, MMPs and their inhibitors have been strongly linked with the process of metastasis, both by the association of increased proteolytic capacity with the metastatic phenotype and functionally as contributors to the process. How MMPs and their inhibitors contributed functionally to metastasis has been more difficult to address experimentally. Many initial conclusions about the mechanistic role of MMPs in metastasis have been derived by inference rather than direct experimentation. Metastasis is an *in vivo* process and is hard to observe directly. Most metastasis assays are end point assays, in which input (numbers, type of cells injected, etc.) and output (numbers of metastases counted at the end of the experiment) are known, while mechanisms by which the input resulted in the output are based on inference. In logically considering steps believed to be required for successful metastasis (see Fig. 1), extravasation from blood vessels in target organs has been assumed to be a difficult, rate-limiting process. Because MMPs are able to degrade proteins that make up blood vessel basement membrane, and because the basement membrane underlying vascular endo-

thelial cells appears to be a clear physical barrier to metastasis, the assumption has been made that the major contribution of MMPs to metastasis is in facilitating extravasation. Mechanistic conclusions have also been based on *in vitro* assays that were thought to be appropriate models for *in vivo* processes. For example, *in vitro* invasion assays through basement membrane proteins (Matrigel) have been used to model extravasation. However, as discussed below, logical inferences about rate-limiting steps *in vivo* do not necessarily hold up to experimental scrutiny, and *in vitro* assays may not model the assumed steps *in vivo*.

A procedure for direct *in vivo* observation of early steps in metastasis has been developed (82). This procedure, intravital videomicroscopy, has provided evidence that suggests that some of our assumptions about mechanisms of metastasis need to be revised, based on evidence obtained from direct observation of the process. From results using this procedure, it can be concluded that the role of MMPs and their inhibitors in metastasis may be different, and more complex, than previously assumed.

Intravital videomicroscopy (IVVM) permits direct observation of the microcirculation *in vivo*. It allows observations to be made on steps in metastasis and the steps in the process that are affected by molecules, such as MMPs, implicated in metastasis [reviewed in (83,84)]. The microvasculature of living experimental animals is observed in real time, and interactions of tumor cells with host tissue can be observed and quantified. Results from a series of experiments by use of this procedure suggest that early steps in metastasis, including destruction of cells in the circulation and extravasation, contribute less to metastatic inefficiency than previously assumed. Rather, the regulation of growth of individual extravasated cells in target tissue appears to be rate limiting. Here, we will review some of the evidence from IVVM, as well as recent findings using other approaches, that suggest that the primary functional contribution of MMPs and their inhibitors in metastasis may be at steps after the extravasation stage.

Previously, it was believed that the majority of cells that escape from a tumor into the circulation were destroyed by hemodynamic forces. However, this belief was not supported by direct observation of intravenously injected cancer cells in chick embryos or mice (85,86). Evidence from IVVM (86) suggests that the majority of injected cells not only survive injection and arrest in a target organ but succeed in extravasating. Metastatic inefficiency thus arises from failure of the majority of extravasated cells to successfully grow in the target organ. Moreover, it also had been assumed that highly metastatic cells are better able to extravasate than are poorly metastatic cells. However, studies with IVVM have not supported this idea. In mouse liver, the timing and steps in extravasation were identical for mammary carcinoma cells of high and low metastatic ability, and the difference between the cell lines manifested itself at the postextravasation growth stage (87). In addition, the ability to extravasate was identical for malignant, ras-transformed NIH 3T3 cells and control fibroblasts (NIH 3T3 and primary mouse embryo fibroblasts), whereas the postextravasation growth behavior of these cells reflected their transformed versus normal phenotypes (88). Together, these studies suggest that extravasation may be a relatively easy process, while rate-limiting steps in metastasis occur after the cells have extravasated.

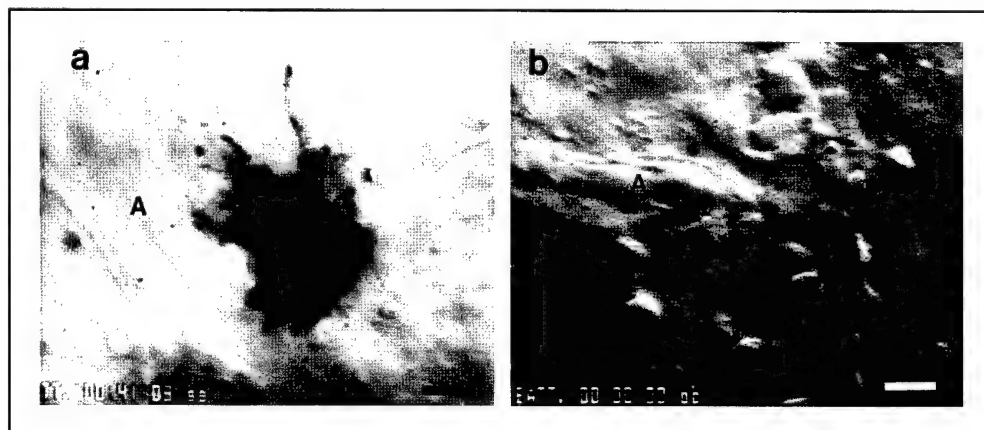
The nature of the contribution of MMPs to the metastatic process was examined directly by IVVM, by using B16F10 mouse melanoma cells engineered to overexpress TIMP-1. The TIMP-1 overexpressing cells had been shown to have markedly reduced metastatic ability (57,89), as measured by end point assays [intravenous injection into mouse or chick embryos and counting of tumors that formed in mouse lung or chick chorio-allantoic membrane, a structure that is structurally and functionally similar to lung, with complete endothelial lining and basement membrane (90)]. Similarly, the TIMP-expressing cells showed reduced *in vitro* invasive ability (76). When these cells were assessed using IVVM, the expectation was that the reduced metastatic ability of the overexpression of TIMP-1 cells would manifest itself in defective extravasation. However, both cell lines were found to extravasate with identical kinetics, with nearly all cells having successfully extravasated by 36 hours after injection (86,91). The reduced metastatic ability of the TIMP-1-expressing cells was manifested by 3 days after injection, when the morphology of micrometastatic colonies was strikingly different from that of control cells (Fig. 3); instead of forming tight, growing colonies in contact with the outer surface of arterioles, the TIMP-1-expressing cells lacked adhesive contacts to other tumor cells and to vessels (where IVVM has shown micrometastases to form) and had abundant stroma between the cells (91). Thus, in this model, overexpression of TIMP-1 had a clear end point effect (i.e., the cells formed fewer, and smaller, metastases) but had no inhibitory effect on extravasation. These findings pointed to a role for MMPs in the regulation of post-extravasation growth. Similar conclusions, which will not be summarized here, can be drawn from other IVVM studies [reviewed in (83,84)].

In light of results from IVVM studies, it is necessary to re-evaluate earlier literature from the perspective of potential effects of MMPs on growth of metastatic lesions as opposed to an effect on extravasation. As discussed previously, experimental and spontaneous metastasis assays that rely on the presence of detectable secondary tumors cannot readily distinguish between these possibilities. However, effects on the growth of the primary tumor can often be detected, and in some studies an analysis of the relative size of secondary tumors has been informative. In early studies by Khokha et al. (54), antisense reduction of TIMP-1 levels in murine fibroblasts allowed these cells to grow into tumors when injected subcutaneously into nude mice; these tumor cells were then capable of metastatic coloni-

zation of the lungs. Recently, the effect of TIMP-1 on the initiation and growth of liver tumors was documented in transgenic mice expressing either sense or antisense TIMP-1 constructs (92). TIMP-1 overexpression inhibited SV40 T-antigen-induced tumor initiation, growth, and angiogenesis, while TIMP-1 reduction resulted in more rapid tumor initiation and progression. TIMP-1 transfection in B16F10 melanoma cells resulted in a decline in primary tumor growth following a subcutaneous injection as well as a reduction in lung colonization following an intravenous injection (57). These results are contrasted with an early study by Schultz et al. (53) also using B16F10 cells, in which an intraperitoneal injection of recombinant TIMP-1 reduced the number of lung colonies but did not alter the size of lung nodules nor did the growth of subcutaneously injected tumors. These authors suggested that the primary effect of TIMP was to inhibit extravasation, a conclusion that was supported by effects on invasion of an amniotic membrane *in vitro*. These apparently contradictory results might be explained by a difference in the experimental protocol or by effects of tumor versus host expression of TIMP-1, since systemic TIMP-1 was elevated following injection of recombinant protein, while in the transfection studies, tumor cell TIMP-1 levels were specifically altered. This possibility is supported by the recent, elegant studies of Soloway et al. (93). Using co-isogenic cells and genetically manipulated mice varying in expression of TIMP-1, these authors demonstrated that lung colonization is influenced by the TIMP-1 genotype of the tumor but not that of the host. Although systemic TIMP-1 may influence extravasation, the initiation and growth of primary tumor cells can be markedly affected by alterations in tumor TIMP-1 levels.

TIMP-2 has also been demonstrated to reduce tumor cell growth as well as metastasis. Transfection or retroviral introduction of TIMP-2 into transformed rat embryo fibroblasts reduced primary tumor growth as well as hematogenous metastasis (75,94). TIMP-2 overexpression reduced the growth of metastatic human melanoma cells injected subcutaneously in immunocompromised mice, although it did not prevent metastasis in this study (95). The growth-inhibitory effect of TIMP-2 was shown to require a three-dimensional collagen matrix and was not observed in gelatin-coated dishes; in the presence of matrix, TIMP-2 expressing melanoma cells demonstrated a reduction in growth rate and assumed a differentiated morphology. Thus, it appears that both TIMP-1 and TIMP-2 can have growth inhibitory effects, and this effect can be dependent on the cel-

Fig. 3. Morphology of nascent micrometastases, 3 days after injection of (a) control B16F10 and (b) tissue inhibitor of metalloproteinases-1 (TIMP-1) overexpressor cells. B16F10 cells formed melanotic, tight perivascular cuffs around arterioles, visible in intravital videomicroscopy by focusing up and down through the lesion, and had a compact tumor morphology. In contrast, TIMP-1 overexpressor cells formed loosely dispersed groups of amelanotic cells, near but not attached to arterioles, lacking homotypic contacts between cells. A = arteriole; bars (a and b) = 20 μ m. Reprinted with permission from Koop et al. (91).



lular environment and on the tumor cells themselves producing the inhibitor.

The effect of TIMPs on the growth of primary tumors and metastatic lesions is further complicated by the observation that TIMP-1 and TIMP-2 also display growth-promoting activity for a variety of cell types (96–100). In fact, TIMP-1 was originally identified as erythroid-potentiating activity, a growth factor for hematopoietic cells of the erythrocyte lineage (101,102). A recent study (103) has dissociated the erythroid-potentiating effect of TIMP-1 from its MMP-inhibitory activity, demonstrating that TIMPs are bifunctional molecules. There are several recent examples of systems in which TIMPs either have no effect or enhance tumor growth and/or metastasis, effects contrary to that expected from its antimetalloproteinase activity. Soloway et al. (93) demonstrated that, although the lack of TIMP-1 expression enhanced lung colonization in two pairs of isogenic cells with wild-type and mutant TIMP-1, in a third pair, lung colonization was reproducibly decreased in the absence of functional TIMP-1. Overexpression of TIMP-1 in the gastrointestinal tract also enhanced development of benign adenomas in a line of transgenic mice carrying a germline mutation in the adenomatous polyposis coli (APC) gene (Heppner KJ, Brown PD, Matrisian LM: manuscript submitted for publication). TIMP-3 overexpression in mouse epidermal cells had no effect on growth, tumorigenicity, or invasion (104). It is possible that the growth-promoting effects of TIMPs are cell type specific, manifest only in cells which, for example, have an appropriate receptor for the domain of TIMP containing the growth-promoting activity. Alternatively, factors such as the relative concentrations of specific TIMPs and/or MMPs and the extracellular environment may all affect how a tumor cell responds to alterations in TIMP expression.

Although the role of MMPs in tumor establishment and growth is difficult to decipher from experiments using multifunctional TIMPs, additional support for such a role comes from studies with the synthetic MMP inhibitors. The first published study (105) with batimastat demonstrated that this compound dramatically reduced tumor burden in an ovarian ascites xenograft. Batimastat caused a delay in growth of the primary tumor and a reduction in the weight of metastases in B16-BL6 melanoma cells (59), and an effect on the regrowth of resected breast tumors was observed following orthotopic injection of human breast cancer cells in nude mice (63). Batimastat also inhibited primary tumor growth in an orthotopic model of colon cancer (62) and of a hemangioma (78). In systems in which the relative size of metastatic nodules was noted, batimastat reduced the size of lung or liver colonies following injection of rat mammary carcinoma or human colorectal cancer cells (60,61). Other MMP inhibitors have also been reported to alter the growth of primary tumors and their metastases *in vivo*, either alone (65,106) or in combination with standard chemotherapeutic agents (66). Batimastat does not alter the growth of tumor cells in plastic culture dishes (105). It is not clear, however, if MMP inhibitors may suppress growth in three-dimensional collagen, as has been observed for TIMP-2 (95). In some cases, the effects of MMP inhibitors on growth *in vivo* may be related to their effects on tumor angiogenesis. MMP inhibitors block angiogenesis as assayed in chick and rodent models of neovascularization (78,107–109). The mechanism of inhibition of tumor growth may reflect

both indirect effects on angiogenesis as well as more direct effects on the growth of tumor cells themselves.

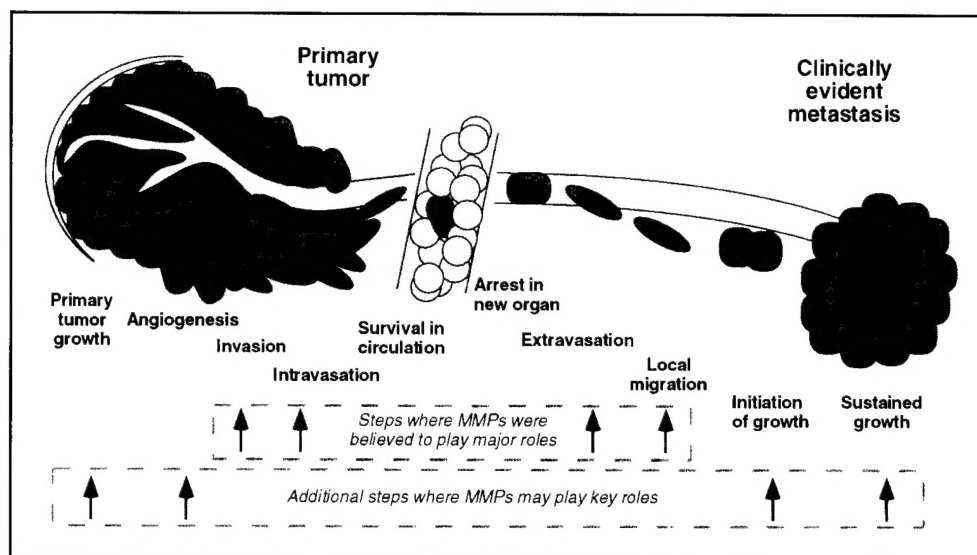
The most definitive evidence for a role for MMPs in tumor cell establishment and growth comes from studies in which levels of a specific MMP were manipulated. Stromelysin-3 was originally isolated from the stroma surrounding malignant breast carcinomas (110). This MMP was overexpressed in human breast cancer cells or removed by antisense RNA from murine fibroblasts, which were then assayed for subcutaneous tumor development in nude mice. Manipulation of stromelysin-3 levels altered the tumorigenicity of the cells but did not alter the growth of established tumors, their invasion, or their metastatic capability (81). Collagenase expression in the skin of transgenic mice resulted in earlier onset and increased numbers of papillomas arising after chemical initiation and promotion (111). Expression of stromelysin-1 in mammary glands of transgenic mice resulted in the development of aggressive malignant mammary tumors (112). In contrast, chemically initiated mammary tumors were actually reduced in other stromelysin-1 transgenic mice (113). However, this effect was determined to be related to an increase in both proliferation and apoptosis in target mammary epithelial cells, a result that is conceptually consistent with a tumor-promoting effect of stromelysin-1 on spontaneous-derived tumors. Taken together, these results suggest that stromelysin expression can promote tumor take and suggest that metalloproteinases may favor cancer cell survival in a tissue environment initially not permissive for tumor growth.

Matrilysin is distinct from other MMPs in that it is expressed in epithelial-derived rather than mesenchymal-derived cells and is expressed in the epithelial component of both benign and malignant stages of many common adult adenocarcinomas (114,115). Genetic manipulation of matrilysin levels in human colon tumor cell lines resulted in an effect on the tumorigenicity of the cells following orthotopic injection into the cecum of nude mice, with little detectable effect on invasive or metastatic ability (80). With the use of matrilysin-deficient mice, the role of matrilysin in the development of benign intestinal adenomas was determined in mice carrying a germline mutation of the APC gene. A significant reduction in both number and size of intestinal adenomas was observed in matrilysin-null mice compared with wild-type control mice (116). Interestingly, the tumors arising in the matrilysin-deficient mice induced gelatinase A in the surrounding stroma, suggesting that MMP activity provided a selective advantage for initiated cells to grow into detectable tumors. In gain-of-function experiments, matrilysin expression in the mammary epithelium of transgenic mice significantly accelerated development of MMTV-neu-induced tumors (Rudolph-Owen LA, Matrisian LM: unpublished results). These results support a role for MMPs, and matrilysin in particular, in the development and growth of early stage tumors.

New View of the Contributions of MMPs to Metastasis

MMPs have long been associated with metastasis, and there is no doubt that they are major functional contributors to the metastatic process. The nature of their contribution originally was assumed to be primarily facilitation of the breakdown of

Fig. 4. Role of metalloproteinases in the metastatic process. Matrix metalloproteinases (MMPs) have classically been associated with steps in the metastatic cascade that involve matrix degradation, including invasion, intravasation, extravasation, and local migration. Evidence discussed in this review expands the role of MMPs to steps involving the growth of the primary tumor, angiogenesis, the initiation of growth at an ectopic site, and the sustained growth of metastatic foci to become clinically detectable tumors.



physical barriers between a primary tumor and distant sites for metastasis. As shown in Fig. 4, these steps include local invasion and intravasation of cancer cells, facilitating their departure from the primary tumor and their access to the lymphatic or blood circulations, and extravasation and local invasion of cancer cells in distant organs, as a first step toward the establishment of secondary tumors. Recent evidence, summarized in this review, suggests that MMPs play a much broader role in metastasis than previously believed, and that action of MMPs at steps both before and after the breakdown of the apparent physical barriers to metastasis may in fact be of greater importance. MMPs and their inhibitors appear to be important regulators of the growth of tumors, both at the primary site and as metastases (Fig. 4). The mechanisms of this growth regulation are not yet fully characterized, but a number of mechanisms are possible. First, MMPs appear to contribute to the initiation of growth, at both primary and secondary sites. One can speculate that this may involve regulation of the growth environment by, for example, regulating access to growth factors from the extracellular matrix surrounding the growing tumor, either directly or via a proteolytic cascade. Similarly, MMPs and their inhibitors appear to regulate the sustained growth of tumors. Beyond the maintenance of an appropriate growth environment, the role of MMPs in angiogenesis is likely important at this stage. Angiogenesis is required for growth of tumors, primary and metastases, beyond small size, and MMPs play a contributory role in regulation of angiogenesis. Details of the mechanisms by which MMPs and their inhibitors contribute to creating an environment that favors the initiation and continued growth of primary and metastatic tumors remain to be elucidated, but are of key importance in cancer therapy. An understanding of the molecular role of MMPs at each of the sequential steps required to produce clinically evident metastases will be important in the design and appropriate use of novel therapeutics designed to combat metastasis.

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Notes

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